12-16-99

UTILITY CONTINUATION PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.: 2997-1-3-1-4

Inventors: William R. Barclay of 7356 Panorama Drive, Boulder, Colorado 80303

Express Mail Label No.: EL417665368US

Title: "A METHOD FOR REDUCING CORROSION IN A FERMENTOR" (As Amended)

Group Art Unit:

Examiner:

Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

This is a Continuation application of pending prior application No. 08/968,628 filed November 12, 1997. The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied, is considered to be part of the disclosure of the accompanying application and is hereby incorporated by reference.

Enclosed for filing with the above-identified utility patent application, please find the following:

1. [X] Copy of Oath/Declaration from the above-referenced pending prior application (37 CFR 1.63(d))
2. [X] Copy on newly executed Oath/Declaration
3. [X] Assignment Papers *(cover sheet & document(s))*
4. [X] Power of Attorney
5. [X] Preliminary Amendment
6. [X] Return Postcard (MPEP 503) *(should be specifically itemized)*
7. [X] A check in the amount of $1,090.00 is enclosed.

FEE CALCULATION:

Cancel in this application original Claims 1 - 37 of the prior application before calculating the filing fee.

	(COL. 1) NO. FILED			(COL. 2*) NO. EXTRA	SMALL ENTITY RATE	SMALL ENTITY FEE		LARGE ENTITY RATE	LARGE ENTITY FEE
BASIC FEE:						$380.00	OR		$760.00
TOTAL CLAIMS:	34	-	20	14	X $9 =	$0.00	OR	X $18 =	$252.00
INDEP. CLAIMS:	4	-	3	1	X $39 =	$0.00	OR	X $78 =	$78.00
____ MULTIPLE DEPENDENT CLAIMS					+ $130 =	$	OR	+$260 =	$0.00
*IF THE DIFFERENCE IN COL. 2 IS LESS THAN ZERO, ENTER "0" IN COL. 2.					TOTAL:	0.00			$1,090.00

OTHER INFORMATION:

1. [X] The Commissioner is hereby authorized to debit any underpayments or credit any overpayment to Deposit Account No. 19-1970.

2. [X] The Commissioner is hereby authorized to charge all required fees for extensions of time under §1.17 to Deposit Account No. 19-1970.

3. [X] The prior application is assigned to OmegaTech Inc..
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Respectfully Submitted,

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Date: 14-DEC-99

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:) Group Art Unit:
)
BARCLAY) Examiner: Not yet assigned
)
Serial No.: Not yet assigned) PRELIMINARY AMENDMENT
)
Filed: Herewith)
)
Atty. File No.: 2997-1-3-1-4)
)
For: "A METHOD FOR REDUCING)
CORROSION IN A FERMENTOR")
(As Amended))

"EXPRESS MAIL" LABEL NUMBER: EL417665368US
DATE OF DEPOSIT: December 14, 1999

I HEREBY CERTIFY THAT THIS PAPER OR FEE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE "EXPRESS MAIL POST OFFICE TO ADDRESSEE" SERVICE UNDER 37 CFR 1.10 ON THE DATE INDICATED ABOVE AND IS ADDRESSED TO THE COMMISSIONER OF PATENTS AND TRADEMARKS, WASHINGTON, D.C. 20231.

TYPED OR PRINTED NAME: Christine Jacquet

SIGNATURE: Christine Jacquet

Box PATENT APPLICATION
Assistant Commissioner
for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Please amend the above-identified patent application as follows:

IN THE TITLE:

At page 1 of the specification, please delete lines 1-3 and substitute in place thereof:

--"A METHOD FOR REDUCING CORROSION IN A FERMENTOR"--.

IN THE SPECIFICATION:

On Page 1, in the section entitled "Cross-Reference to Related Applications," please delete lines 6-19 and substitute in place thereof:

-- This application is continuation of U.S. Patent Application Serial No. 08/968,628, filed November 12, 1997, which is a continuation of U.S. Patent Application Serial No. 08/461,137, filed June 5, 1995, which issued as U.S. Patent No. 5,688,500 on November 18, 1997, which is a continuation of U.S. Patent Application Serial No. 08/292,490, filed August 18, 1994, which issued as U.S. Patent No. 5,518,918 on May 21, 1996, which is a continuation of U.S. Patent Application Serial No. 07/962,522, filed October 16, 1992, which issued as U.S. Patent No. 5,340,742 on August 23, 1994.

This application is also related to, but does not claim priority from, U.S. Patent Application Serial No. 07/911,760, filed July 10, 1992, which issued as U.S. Patent No. 5,340,594 on August 23, 1994, which is a continuation of U.S. Patent Application Serial No. 07/580,778, filed September 11, 1990, which issued as U.S. Patent No. 5,130,242 on July 14, 1992, which is a continuation-in-part of U.S. Patent Application Serial No. 07/439,093, filed November 17, 1989, which was previously expressly abandoned, which is a continuation-in-part of U.S. Patent Application Serial No. 07/241,410, filed September 7, 1988, which was previously expressly abandoned.

U.S. Patent Application Serial No. 08/968,628 is also related to, but the present application does not claim priority from, the following applications: U.S. Patent Application Serial No. 08/968,628 is also a continuation-in-part of U.S. Patent Application Serial No. 08/483,477, filed June 7, 1995, now U.S. Patent No. 5,698,244, issued December 16, 1997, which is continuation-in-part of U.S. Patent Application Serial No. 08/292,736, filed August 18, 1994, now U.S. Patent No. 5,656,319, issued August 12, 1997, which is a continuation of U.S. Patent application Serial No. 07/911,760, filed July 10, 1992, now U.S. Patent No. 5,340,594, issued August 23, 1994, which is a continuation of U.S. Patent application Serial No. 07/580,778, filed September 11, 1990, now U.S. Patent No. 5,130,242, issued July 14, 1992, which is a continuation-in-part application of U.S. Patent application Serial No. 07/439,093, filed November 17, 1989, now abandoned, which is a continuation-in-part of U.S. Patent Application Serial No. 07/241,410, filed September 7, 1988, now abandoned.

Furthermore, U.S. Patent Application Serial No. 08/968,628 is also related to, but the present application does not claim priority from, the following applications: U.S. Patent Application Serial No. 08/968,628 is also a continuation-in-part of U.S. Patent Application Serial No. 08/918,325, filed August 26, 1997, which is

a continuation of U.S. Patent Application Serial No. 08/483,477, filed June 7, 1995, now U.S. Patent No. 5,698,244, issued December 16, 1997.

All of the above patents and patent applications are incorporated herein by reference in their entirety.--

IN THE CLAIMS:

Please cancel all pending claims without prejudice or disclaimer of the subject matter thereof and add the following claims, renumbered as Claims 38-71.

38. (New) A method for reducing corrosion of a fermentor during growth of microorganisms in a saline fermentation medium, said method comprising:

growing the microorganisms in the fermentor comprising a culture medium in which one of the primary inorganic ions is sodium which is provided in the form of a non-chloride sodium salt, wherein the culture medium contains a chloride concentration of less than about 3 grams chloride per liter of culture medium, and wherein the culture medium containing the non-chloride sodium salt as the primary source of sodium results in reduced fermentor corrosion compared to the culture medium containing sodium chloride as the primary source of sodium.

39. (New) The method of Claim 38, wherein less than about 50% of the sodium in the fermentation medium is supplied as sodium chloride.

40. (New) The method of Claim 38, wherein the non-chloride sodium salt is selected from the group consisting of soda ash, sodium carbonate, sodium bicarbonate, sodium sulfate and mixtures thereof.

41. (New) The method of Claim 38, further comprising the step of maintaining the proper pH of the fermentation medium.

42. (New) The method of Claim 38, wherein the non-chloride sodium salt comprises sodium sulfate.

43. (New) The method of Claim 42, wherein the concentration of said sodium sulfate is greater than about 1 g/L.

44. (New) The method of Claim 42, wherein the concentration of said sodium sulfate is between about 1 g/L and about 50 g/L.

45. (New) The method of Claim 42, wherein the concentration of said sodium sulfate is between about 2 g/L and about 25 g/L.

46. (New) The method of Claim 38, wherein the culture medium contains the chloride concentration of less than about 500 mg chloride per liter of culture medium.

47. (New) The method of Claim 38, wherein the microorganisms are obtained from a marine or inland saline environment.

48. (New) The method of Claim 38, wherein the microorganisms are selected from the group consisting of algae, yeasts, bacteria, fungi and mixtures thereof.

49. (New) The method of Claim 38, wherein the microorganisms are selected from the group consisting of microorganisms which are capable of growth at a salinity level which results in a conductivity of from about 5 mmho/cm to about 40 mmho/cm.

50. (New) The method of Claim 38, wherein the microorganisms are selected from the group consisting of microorganisms which are capable of growth in 60% seawater or 60% artificial seawater.

51. (New) The method of Claim 38, wherein the microorganisms are selected from the group consisting of *Thraustochytrium*, *Schizochytrium* and mixtures thereof.

52. (New) The method of Claim 38, wherein the microorganisms have all of the identifying characteristics of an organism selected from the group consisting of ATCC Nos. 20888 and 20889, and mutants thereof, wherein said mutants have an omega-3 HUFA content of at least about 0.5% dry weight.

53. (New) A method for reducing corrosion of a fermentor during growth of microorganisms in a saline fermentation medium, said method comprising:

growing the microorganisms in the fermentor comprising a culture medium in which one of the primary inorganic ions is sodium

which is provided in the form of a non-chloride sodium salt, wherein the non-chloride sodium salt is selected from the group consisting of soda ash, sodium carbonate, sodium bicarbonate, sodium sulfate and mixtures thereof, and wherein the culture medium containing the non-chloride sodium salt as the primary source of sodium results in reduced fermentor corrosion compared to the culture medium containing sodium chloride as the primary source of sodium.

54. (New) The method of Claim 53, wherein the culture medium contains a chloride concentration of less than about 3 grams chloride per liter of culture medium.

55. (New) The method of Claim 53, wherein the culture medium contains a chloride concentration of less than about 500 mg chloride per liter of culture medium.

56. (New) The method of Claim 53, wherein the non-chloride sodium salt comprises sodium sulfate.

57. (New) The method of Claim 56, wherein the concentration of said sodium sulfate is greater than about 1 g/L.

58. (New) The method of Claim 53, wherein the microorganisms are obtained from a marine or inland saline environment.

59. (New) The method of Claim 58, wherein the microorganisms are selected from the group consisting of microorganisms which are capable of growth at a salinity level which results in a conductivity of from about 5 mmho/cm to about 40 mmho/cm.

60. (New) The method of Claim 58, wherein the microorganisms are selected from the group consisting of microorganisms which are capable of growth in 60% seawater or 60% artificial seawater.

61. (New) The method of Claim 53, wherein the microorganisms are selected from the group consisting of *Thraustochytrium*, *Schizochytrium* and mixtures thereof.

62. (New) The method of Claim 53, wherein the microorganisms have all of the identifying characteristics of an organism selected from the group consisting of ATCC Nos. 20888 and 20889, and mutants thereof, wherein said mutants have an omega-3 HUFA content of at least about 0.5% dry weight.

63. (New) A method for reducing corrosion of a fermentor during growth of microorganisms in a saline fermentation medium, said method comprising:

growing the microorganisms in the fermentor comprising a culture medium in which one of the primary inorganic ions is sodium which is provided in the form of a non-chloride sodium salt comprising sodium sulfate, wherein the culture medium contains a chloride concentration of less than about 3 grams chloride per

liter of culture medium, and wherein the culture medium containing the non-chloride sodium salt as the primary source of sodium results in reduced fermentor corrosion compared to the culture medium containing sodium chloride as the primary source of sodium.

64. (New) The method of Claim 63, wherein less than about 50% of the sodium in the fermentation medium is supplied as sodium chloride.

65. (New) The method of Claim 63, wherein the concentration of said sodium sulfate is greater than about 1 g/L.

66. (New) The method of Claim 63, wherein the microorganisms are selected from the group consisting of *Thraustochytrium*, *Schizochytrium* and mixtures thereof.

67. (New) The method of Claim 63, wherein the microorganisms have all of the identifying characteristics of an organism selected from the group consisting of ATCC Nos. 20888 and 20889, and mutants thereof, wherein said mutants have an omega-3 HUFA content of at least about 0.5% dry weight.

68. (New) A method for reducing corrosion of a fermentor during growth of microorganisms in a saline fermentation medium, wherein the microorganisms are selected from the group consisting of *Thraustochytrium*, *Schizochytrium* and mixtures thereof, said method comprising:

growing the microorganisms in the fermentor comprising a culture medium in which one of the primary inorganic ions is sodium which is provided in the form of a non-chloride sodium salt comprising sodium sulfate, wherein the culture medium contains a chloride concentration of less than about 3 grams chloride per liter of culture medium, and wherein the culture medium containing the non-chloride sodium salt as the primary source of sodium results in reduced fermentor corrosion compared to the culture medium containing sodium chloride as the primary source of sodium.

69. (New) The method of Claim 68, wherein less than about 50% of the sodium in the fermentation medium is supplied as sodium chloride.

70. (New) The method of Claim 68, wherein the concentration of said sodium sulfate is greater than about 1 g/L.

71. (New) The method of Claim 68, wherein said microorganisms have all of the identifying characteristics of an organism selected from the group consisting of ATCC Nos. 20888 and 20889, and mutants thereof, wherein said mutants have an omega-3 HUFA content of at least about 0.5% dry weight.

REMARKS

This present application is a continuation of U.S. Patent Application Serial No. 08/968,628, filed November 12, 1997.

A check in the amount of $1090.00 is enclosed as payment of the filing fee for this continuation application. Please debit any underpayment or credit any overpayment to Deposit Account No. 19-1970.

Respectfully submitted,

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PROCESS FOR THE HETEROTROPHIC PRODUCTION OF MICROBIAL PRODUCTS WITH HIGH CONCENTRATIONS OF OMEGA-3 HIGHLY UNSATURATED FATTY ACIDS

Cross-Reference to Related Applications

This application is a continuation-in-part of copending and commonly assigned U.S. patent application Serial No. 07/911,760, filed July 10, 1992 which is incorporated herein in its entirety by reference which is a divisional of U.S. patent application Serial No. 07/580,778, filed September 11, 1990 which is incorporated herein in its entirety which issued as U.S. Patent No. 5,130,242 which is a continuation-in-part of copending and commonly assigned U.S. patent application Serial No. 07/439,093, filed November 17, 1989 which is incorporated herein in its entirety by reference which is a continuation-in-part of U.S. Patent Application Serial No. 07/241,410, filed September 7, 1988 which was previously expressly abandoned.

Field of the Invention

The field of this invention relates to heterotrophic organisms and a process for culturing them for the production of lipids with high concentrations of omega-3 highly unsaturated fatty acids (HUFA) suitable for human and animal consumption as food additives or for use in pharmaceutical and industrial products.

Background of the Invention

Omega-3 highly unsaturated fatty acids (HUFAs) are of significant commercial interest in that they have been

recently recognized as important dietary compounds for preventing arteriosclerosis and coronary heart disease, for alleviating inflammatory conditions and for retarding the growth of tumor cells. These beneficial effects are a result both of omega-3 HUFAs causing competitive inhibition of compounds produced from omega-6 fatty acids, and from beneficial compounds produced directly from the omega-3 HUFAs themselves (Simopoulos et al., 1986). Omega-6 fatty acids are the predominant HUFAs found in plants and animals. Currently, a commercially available dietary source of omega-3 HUFAs is from certain fish oils which can contain up to 20-30% of these fatty acids. The beneficial effects of these fatty acids can be obtained by eating fish several times a week or by daily intake of concentrated fish oil. Consequently large quantities of fish oil are processed and encapsulated each year for sale as a dietary supplement. However, there are several significant problems with these fish oil supplements, including bioaccumulation of fat-soluble vitamins and high levels of saturated and omega-6 fatty acids, both of which can have deleterious health effects.

Another source of omega-3 HUFAS is the microflora *Thraustochytrium* and *Schizochytrium* which are discussed in detail in related U.S. Patent No. 5,130,242. These microflora have the advantages of being heterotrophic and capable of high levels of omega-3 HUFA production. There still exists a need however for improved methods for

fermentation of these microflora and identification of improved uses of the microflora product.

Brief Summary of the Invention

The present invention is directed to a new process for growing the microflora *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, which includes the growing of the microflora in a culture medium containing non-chloride containing sodium salts, particularly including sodium sulfate. More particularly, a significant portion of the sodium requirements of the fermentation are supplied as a non-chloride containing sodium salt. The present process is particularly useful in commercial production because the chloride content in the medium can be significantly reduced, thereby avoiding the corrosive effects of chloride on fermentation equipment. In addition, the present invention is particularly useful for production of food products for use in aquaculture because *Thraustochytrium* and *Schizochytrium* cultured in such media form much smaller clumps than those cultured in high chloride media and are thus more available as a food source for larval shrimp. In particular, *Thraustochytrium* and *Schizochytrium* cultured in medium containing sodium sulfate can have cell aggregates of an average size of less than about 150 microns in diameter.

A further embodiment of the present invention is the production of a microflora biomass comprising *Thraustochytrium*, *Schizochytrium*, and mixtures thereof which

have an average cell aggregate size of less than about 150 microns. The microflora biomass is useful for aquaculture and in particular, for feeding larval shrimp because the microflora have the primary feed advantages required for shrimp of a high sterol content and a high omega-3 highly unsaturated fatty acid (HUFA) content. Additionally, because of the small cell aggregate size, the microflora can be eaten by the larval shrimp, brine shrimp, rotifers, and mollusks. The present invention further includes a process for the production of these organisms which includes feeding *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, having an average cell size of less than about 150 microns to them.

A further embodiment of the present invention is directed to a food product which is comprised of microflora selected from the group consisting of *Thraustochytrium*, *Schizochytrium*, and mixtures thereof and an additional component selected from the group consisting of flaxseed, rapeseed, soybean, avocado meal, and mixtures thereof. A particular advantage of this food product is that it has a high long chain omega-3 fatty acid content and a high short chain omega-3 fatty chain content from the additional component. In a further embodiment, the food product is produced by extrusion. The extrusion process involves mixing the microflora with the additional component, thereby reducing the moisture content of the food product. The food product is then extruded under heat, thus driving off a significant portion of the reduced moisture. The remaining

amount of the original moisture content is readily removed by air drying or short baking times, thereby reducing the overall energy requirements of drying and the potential degradation of the omega-3 HUFA's by extended drying at high temperatures.

Brief Description of the Figures

Fig. 1 is a graphical representation of HUFA production in newly isolated strains of the invention, represented by ■, and previously isolated strains represented by +. Each point represents a strain, the position of each point is determined by the percent by weight of total fatty acids which were omega-3 HUFAs (abscissa) and the percent by weight of total fatty acids which were omega-6 fatty acids (ordinate). Only those strains of the invention were plotted wherein less than 10.6% (w/w) of total fatty acids were omega-6 and more than 67% of total fatty acids were omega-3.

Fig. 2 is a graphical representation of HUFA production in newly isolated strains of the invention, represented by ■, and previously isolated strains, represented by +. Each point represents a strain, the position of each point is determined by the percent by weight of total fatty acids which were omega-3 HUFAs (abscissa) and percent of weight of total fatty acids which were eicosapentaenoic acid (EPA C20:5n-3) (ordinate). Only those strains of the invention were plotted wherein more than 67% (w/w) of total fatty acids were omega-3 and more than 7.8% (w/w) of total fatty acids were C20:5n-3.

Fig. 3 is a graphical representation of omega-3 HUFA composition in newly isolated strains of the invention, represented by □, and previously isolated strains, represented by +. Each point represents a separate strain. Values on the abscissa are weight fraction of total omega-3 HUFAs which were C20:5n-3 and on the ordinate are weight fraction of total omega-3 fatty highly unsaturated acids which were C22:6n-3. Only strains of the invention were plotted having either a weight fraction of C20:5n-3 28% or greater, or a weight fraction of C22:6n-3 greater than 93.6%.

Fig. 4 is a graph showing growth of various newly isolated strains of the invention and previously isolated strains, at 25°C and at 30°C. Growth rates are normalized to the growth rate of strain U-30 at 25°C. Previously isolated strains are designated by their ATCC accession numbers.

Fig. 5 is a graph of total yields of cellular production after induction by nitrogen limitation. Each of ash-free dry weight, total fatty acids and omega-3 HUFAs, as indicated, was plotted, normalized to the corresponding value for strain 28211. All strains are identified by ATCC accession numbers.

Fig. 6 is a graph of fatty acid yields after growth in culture media having the salinity indicated on the abscissa. Strains shown are newly isolated strains S31 (ATCC 20888) (□) and U42-2 (ATCC 20891) (+) and previously isolated strains, ATCC 28211 (◇) and ATCC 28209 (Δ). Fatty acid yields are plotted as relative yields normalized to an

arbitrary value of 1.00 based on the average growth rate exhibited by S31 (ATCC 20888) (□) over the tested salinity range.

Fig. 7 is a graph of increases in the omega-3 HUFA content of the total lipids in the brine shrimp, *Artemia salina*, fed Thraustochytrid strain (ATCC 20890) isolated by the method in Example 1. EPA = C20:5n-3; DHA = C22:5n-3.

Fig. 8 is a graph of increases in the omega-3 HUFA content of the total lipids in the brine shrimp, *Artemia salina*, fed Thraustochytrid strain (ATCC 20888) isolated by the method in Example 1. EPA = C20:5n-3; DHA = C22:5n-3.

Detailed Description of the Preferred Embodiments

For purposes of definition throughout the application, it is understood herein that a fatty acid is an aliphatic monocarboxylic acid. Lipids are understood to be fats or oils including the glyceride esters of fatty acids along with associated phosphatides, sterols, alcohols, hydrocarbons, ketones, and related compounds.

A commonly employed shorthand system is used in this specification to denote the structure of the fatty acids (e.g., Weete, 1980). This system uses the letter "C" accompanied by a number denoting the number of carbons in the hydrocarbon chain, followed by a colon and a number indicating the number of double bonds, i.e., C20:5, eicosapentaenoic acid. Fatty acids are numbered starting at the carboxy carbon. Position of the double bonds is indicated by adding the Greek letter delta (Δ) followed by

the carbon number of the double bond; i.e., C20:5omega-3$\Delta^{5,8,11,14,17}$. The "omega" notation is a shorthand system for unsaturated fatty acids whereby numbering from the carboxy-terminal carbon is used. For convenience, n-3 will be used to symbolize "omega-3," especially when using the numerical shorthand nomenclature described herein. Omega-3 highly unsaturated fatty acids are understood to be polyethylenic fatty acids in which the ultimate ethylenic bond is 3 carbons from and including the terminal methyl group of the fatty acid. Thus, the complete nomenclature for eicosapentaenoic acid, an omega-3 highly unsaturated fatty acid, would be C20:5n-3$\Delta^{5,8,11,14,17}$. For the sake of brevity, the double bond locations ($\Delta^{5,8,11,14,17}$) will be omitted. Eicosapentaenoic acid is then designated C20:5n-3, Docosapentaenoic acid (C22:5n-3$\Delta^{7,10,13,16,19}$) is C22:5n-3, and Docosahexaenoic acid (C22:6n-3$\Delta^{4,7,10,13,16,19}$) is C22:6n-3. The nomenclature "highly unsaturated fatty acid" means a fatty acid with 4 or more double bonds. "Saturated fatty acid" means a fatty acid with 1 to 3 double bonds.

A collection and screening process has been developed to readily isolate many strains of microorganisms with the following combination of economically desirable characteristics for the production of omega-3 HUFAs: 1) capable of heterotrophic growth; 2) high content of omega-3 HUFAs; 3) unicellular; 4) preferably low content of saturated and omega-6 HUFAs; 5) preferably nonpigmented, white or essentially colorless cells; 6) preferably thermotolerant (ability to grow at temperatures above 30°C); and 7)

preferably euryhaline (able to grow over a wide range of salinities, but especially at low salinities). This process is described in detail in related U.S. Patent No. 5,130,242.

Using the collection and screening process, strains of unicellular microflora can be isolated which have fatty acid contents up to about 45% total cellular dry weight percent (%dwt), and which exhibit growth over a temperature range from 15-48°C and grow in a very low salinity culture medium. Many of the very high omega-3 strains are very slow growers. Strains which have been isolated by the method outlined above, and which exhibit rapid growth, good production and high omega-3 HUFA content, have omega-3 unsaturated fatty acid contents up to approximately 12% dwt.

One aspect of the present invention is the growth of *Thraustochytrium*, *Schizochytrium*, and mixtures thereof with high omega-3 HUFA content, in fermentation medium containing non-chloride containing sodium salts and preferably sodium sulfate. More particularly, a significant portion of the sodium requirements of the fermentation are supplied as non-chloride containing sodium salts. For example, less than about 75% of the sodium in the fermentation medium is supplied as sodium chloride, more preferably less than about 50% and more preferably less than about 25%. A particular advantage of the present invention is that the medium provides the source of sodium needed by the microflora to grow in the absence of a significant amount of chloride which can corrode the vessel in which the microflora are being grown and other fermentation or downstream processing

equipment. It has been surprisingly found that microflora of the present invention can be grown at chloride concentrations of less than about 3 gl/l, more preferably less than about 500 mg/l, more preferably less than about 250 mg/l and more preferably between about 60 mg/l and about 120 mg/l while still attaining high yields of biomass per sugar of about 50% or greater. As discussed below, an additional advantage of the present invention is the production of microflora that are high in omega-3 HUFA content but have a small enough cell aggregate size to be consumed by larval shrimp, brine shrimp, rotifers and mollusks.

Non-chloride containing sodium salts can include soda ash (a mixture of sodium carbonate and sodium oxide), sodium carbonate, sodium bicarbonate, sodium sulfate and mixtures thereof, and preferably include sodium sulfate. Soda ash, sodium carbonate and sodium bicarbonate tend to increase the pH of the fermentation medium, thus requiring control steps to maintain the proper pH of the medium. The concentration of sodium sulfate is effective to meet the salinity requirements of the microflora, preferably the sodium concentration is (expressed as g/l of Na) is greater than about 1.0 g/l, more preferably between about 1.0 g/l and about 50.0 g/l and more preferably between about 2.0 g/l and about 25 g/l.

It has been surprisingly found that fermentation of the strains in the presence of a non-chloride containing sodium salt and particularly, sodium sulfate limits the cell

aggregate size of the strains to less than about 150 microns, preferably less than about 100 microns, and more preferably less than about 50 microns. As used herein, the term cell aggregate size refers to the approximate average diameter of clumps or aggregates of cells in a fermentation medium of a microfloral culture. Typically, greater than about 25 percent of the cell aggregates in a microfloral culture have cell aggregate size below the average size, more preferably greater than about 50 percent and more preferably greater than about 75 percent. Microfloral cells produced in accordance with the present invention meet cell aggregate size parameters described above while in fermentation medium as well as after freezing and/or drying of the biomass if resuspended in liquid or physically agitated, such as by a blender or vortexer. The present process is particularly important for microflora which replicate by successive bipartition (wherein a single cell replicates by dividing into two cells which each divide into two more, etc.) because as cells repeatedly and rapidly undergo this process, the cells tend to clump forming multi-cell aggregates which are often outside the cell aggregate size parameters identified above. *Schizochytrium* replicate by successive bipartition and by forming sporangia which release zoospores. *Thraustochytrium*, however, replicate only by forming sporangia and releasing zoospores. For *Thraustochytrium* which replicate by sporangia/zoospore formation, clumping can be a problem as well, particularly because even though the number of cells in an aggregate may not be as great as

aggregates formed by successive bipartition, the individual cell sizes of *Thraustochytrium* tend to be larger, and thus, clumps of a small number of cells are larger. However, one deposited strain of *Thraustochytrium*, ATCC 26185, has been identified which does not exhibit significant aggregation.

In another aspect of the present invention, it has been found that by restricting the oxygen content of the fermentation medium during the growth of *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, the lipid content of the strains can be increased. The optimum oxygen concentration for lipid production can be determined for any particular microflora by variation of the oxygen content of the medium. In particular, the oxygen content of the fermentation medium is maintained at an oxygen content of less than about 40% of saturation and preferably between about 5% of saturation and about 40% of saturation.

Growth of the strains by the invention process can be effected at any temperature conducive to satisfactory growth of the strains; for example, between about 5°C and about 48°C, preferably between about 15°C and about 40° C, and more preferably between about 25°C and about 35°C. The culture medium typically becomes more alkaline during the fermentation if pH is not controlled by acid addition or buffers. The strains will grow over a pH range from 5.0-11.0 with a preferable range of about 6.0-8.5.

Various fermentation parameters for inoculating, growing and recovering microflora are discussed in detail in U.S. Patent No. 5,130,242. The biomass harvested from a

fermentation run can be dried (e.g., spray drying, tunnel drying, vacuum drying, or a similar process) and used as a feed or food supplement for any animal whose meat or products are consumed by humans. Similarly, extracted omega-3 HUFAs can be used as a feed or food supplement. Alternatively, the harvested and washed biomass can be used directly (without drying) as a feed supplement. To extend its shelf life, the wet biomass can be acidified (approximate pH = 3.5-4.5) and/or pasteurized or flash heated to inactivate enzymes and then canned, bottled or packaged under a vacuum or non-oxidizing atmosphere (e.g., N_2 or CO_2). The term "animal means any organism belonging to the kingdom Animalia and includes, without limitation, any animal from which poultry meat, seafood, beef, pork or lamb is derived. Seafood is derived from, without limitation, fish, shrimp and shellfish. The term "products" includes any product other than meat derived from such animals, including, without limitation, eggs or other products. When fed to such animals, omega-3 HUFAs in the harvested biomass or extracted omega-3 HUFAs are incorporated into the flesh, eggs or other products of such animals to increase the omega-3 HUFA content thereof.

A further embodiment of the present invention is the use of the harvested biomass as a food product for larval shrimp, brine shrimp, rotifers and mollusks and in particular, larval shrimp. During the larval stage of development, shrimp larvae are unable to use some food sources because the food source is too large. In particular, at certain stages of development, shrimp larvae are unable

to use a food source having a diameter greater than about 150 microns. Thus, microflora grown in fermentation medium containing a non-chloride sodium salt, and particularly sodium sulfate, as broadly discussed above, are suitable for use as a shrimp food product. As discussed above, microflora grown under such conditions typically have a cell aggregate size less than about 150 microns, preferably less than about 100 microns, and more preferably less than about 50 microns.

A further advantage of the use of microflora of the present invention as a food source for shrimp is that such microflora have a significant sterol content including cholesterol, which is a primary feed requirement for shrimp. The microflora of the present invention typically have a sterol content of preferably at least about 0.1% ash-free dry weight (afdw), more preferably at least about 0.5% afdw, and even more preferably at least about 1.0% afdw. In addition, the microflora of the present invention typically have a cholesterol content of preferably at least about 15% of the total sterol content, more preferably at least about 25% of the total sterol content, and even more preferably at least about 40% of the total sterol content. Further, the microfloral biomass of the present invention also provide shrimp with additional nutritional requirements such as omega-6 fatty acids, protein, carbohydrates, pigments and vitamins.

The microbial product of the present invention is of value as a source of omega-3 HUFAs for fish, shrimp and other products produced by aquaculture. The product can be used

as a food product as described above for shrimp; or added directly as a supplement to the feed for shrimp and fish, generally; or it can be fed to brine shrimp or other live feed organisms intended for consumption by an aquacultured organism. The use of such microflora in this manner enables the shrimp farmer to obtain significantly higher growth rates and/or survival rates for larval shrimp and to produce post-larval shrimp which are more hardy and robust.

For most feed applications, the fatty acid content of the harvested cells will be approximately 15-50% dwt with the remaining material being largely protein and carbohydrate. The protein can contribute significantly to the nutritional value of the cells as several of the strains that have been evaluated have all of the essential amino acids and would be considered a nutritionally balanced protein.

A further embodiment of the present invention is the production of a food product using the *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, of the present invention, combined with an additional component selected from the group consisting of rapeseed, flaxseed, soybean and avocado meal. A particular advantage of this embodiment is that the food product contains both short chain omega-3 HUFAs from the additional component and long chain omega-3 HUFAs from the microflora. Food products having flaxseed, rapeseed, soybeans and avocado meal are known to be useful for supplying a source of short chain omega-3 HUFAs and for additionally supplying a source of short chain omega-3 HUFAs,

which can be elongated by the humans and animals that ingest them. Such food products, however, have the disadvantages of having high choline contents from the additional component, which can form primary amines and result in an unpleasant fish smell; and toxic compounds from the additional component, which at high levels can, for example, inhibit the laying of eggs by hens or cause animals to go off of their feed. As such, the food product of the present invention has the advantage of a lowered flaxseed, rapeseed, soy bean or avocado meal content because the organism ingesting the food product does not need high levels of short chain omega-3 HUFAs for the purpose of converting them to long chain HUFAs. Thus, the lowered content of the flaxseed and rapeseed of the food product results in lowered amounts of choline and/or inhibitory toxic compounds present in the food product.

The amount of *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, used in the food product can range from between about 5% to about 95% by weight. The additional component can be present in the food product at a range of between about 5% to about 95% by weight. Additionally, the food product can include other components as well, including grains, supplements, vitamins, binders and preservatives.

In a preferred embodiment, the above food product is produced using an extrusion process. The extrusion process involves mixing the microflora with the additional component, thereby reducing the moisture in the microfloral biomass by the amount of the additional component mixed. The food

product is extruded under heat, thus removing further moisture from the food product. The resulting product which has a low moisture content can be air dried or dried by relatively short baking times thereby reducing the overall energy requirements of drying and the potential degradation of omega-3 HUFAs due to long time periods at high temperatures. In addition, heat from the extrusion process can degrade some of the unwanted toxic compounds commonly found in the additional component which can, for example, inhibit egg laying by hens or cause animals to go off of their feed.

The present invention will be described in more detail by way of working examples. Species meeting the selection criteria described above have not been described in the prior art. By employing these selection criteria, over 25 potentially promising strains have been isolated from approximately 1000 samples screened. Out of the approximate 20,500 strains in the American Type Culture Collection (ATCC), 10 strains were later identified as belonging to the same taxonomic group as the strains isolated. Those strains still viable in the Collection were procured and used to compare with strains isolated and cultured by the disclosed procedures. The results of this comparison are presented in Examples 4 and 5 below.

The most recent taxonomic theorists place Thraustochydrids with the algae or algae-like protists. All of the strains of unicellular microorganisms disclosed and claimed herein are members of the order Thraustochytriales

(Order: Thraustochytriales; Family: Thraustochytriaceae; Genus: *Thraustochytrium* or *Schizochytrium*). For general purposes of discussion herein, these microorganisms will be called microflora to better denote their uncertain exact taxonomic position.

The novel strains identified below were deposited under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure. All restrictions on the availability to the public of the materials so deposited will be irrevocably removed upon the granting of a patent. Each deposit will be stored for a period of at least five years after the most recent request for the furnishing of a sample of the deposited microorganism is received by the American Type Culture Collection (ATCC), and, in any case, for a period of at least 30 years after the date of the deposit.

Preferred microorganisms of the present invention have all of the identifying characteristics of the deposited strains and, in particular, the identifying characteristics of being able to produce omega-3 HUFAs as described herein and having cell aggregate size characteristics when cultured under conditions as described herein. In particular, the preferred microorganisms of the present invention refer to the following deposited microorganisms and mutants thereof.

Strain	ATCC No.	Deposit Date
Schizochytrium S31	20888	8/8/88
Schizochytrium S8	20889	8/8/88

The present invention, while disclosed in terms of specific organism strains, is intended to include all such methods and strains obtainable and useful according to the teachings disclosed herein, including all such substitutions, modification, and optimizations as would be available expedients to those of ordinary skill in the art.

The following examples and test results are provided for the purposes of illustration and are not intended to limit the scope of the invention.

EXAMPLES

Example 1. Collection and Screening

A 150ml water sample was collected from a shallow, inland saline pond and stored in a sterile polyethylene bottle. Special effort was made to include some of the living plant material and naturally occurring detritus (decaying plant and animal matter) along with the water sample. The sample was placed on ice until return to the laboratory. In the lab, the water sample was shaken for 15-30 seconds, and 1-10ml of the sample was pipetted or poured into a filter unit containing 2 types of filters: 1) on top, a sterile 47mm diameter Whatman #4 filter having a pore size about 25μm; and 2) underneath the Whatman filter, a 47mm diameter polycarbonate filter with about 1.0μm pore size. Given slight variations of nominal pore sizes for the filters, the cells collected on the polycarbonate filter range in size from about 1.0μm to about 25μm.

The Whatman filter was removed and discarded. The polycarbonate filter was placed on solid F-1 media in a petri plate, said media consisting of (per liter): 600ml seawater (artificial seawater can be used), 400ml distilled water, 10g agar, 1g glucose, 1g protein hydrolysate, 0.2g yeast extract, 2ml 0.1 M KH_2PO_4, 1ml of a vitamin solution (A-vits) (Containing 100mg/l thiamine, 0.5mg/l biotin, and 0.5mg/l cyanocobalamin), 5ml of a trace metal mixture (PII metals, containing per liter: 6.0g Na_2EDTA, 0.29g $FeCl_36H_2O$, 6.84g H_3BO_3, 0.86 $MnCl_24H_2O$, 0.06g $ZnCl_2$, 0.026g $CoCl_26H_2O$, (0.052g

$NiSO_4H_2O$, 0.002g $CuSo_45H_2O$, and 0.005g $Na_2MoO_42H_2O$, and 500mg each of streptomycin sulfate and penicillin-G. The agar plate was incubated in the dark at 30°C. After 2-4 days numerous colonies appeared on the filter. Colonies of unicellular microflora (except yeast) were picked from the plate and restreaked on a new plate of similar media composition. Special attention was made to pick all colonies consisting of colorless white cells. The new plate was incubated at 30°C and single colonies picked after a 2-4 day incubation period. Single colonies were then picked and placed in 50ml of liquid medium containing the same organic enrichments as in the agar plates. These cultures were incubated for 2-4 days at 30°C on a rotary shaker table (100-200 rpm). When the cultures appeared to reach maximal density, 20-40ml of the culture was harvested, centrifuged and lyophilized. The sample was then analyzed by standard, well-known gas chromatographic techniques (e.g., Lepage and Roy, 1984) to identify the fatty acid content of the strain. Those strains with omega-3 HUFAs were thereby identified, and cultures of these strains were maintained for further screening.

Using the collection and screening process outlined above, over 150 strains of unicellular microflora have been isolated which have high omega-3 HUFA contents as a percent of total fatty acids and which exhibit growth over a temperature range from 15-48°C. Strains can also be isolated which have less than 1% (as % of total fatty acids) of the undesirable C20:4n-6 and C22:5n-6 HUFAs for some

applications. Strains with high omega-6 content can also be isolated. Strains of these microflora can be repeatedly isolated from the same location using the procedure outlined above. A few of the newly isolated strains have very similar fatty acid profiles. The possibility that some are duplicate isolates of the same strain cannot be ruled out at present. Further screening for other desirable traits such as salinity tolerance or ability to use a variety of carbon and nitrogen sources can then be carried out using a similar process.

Example 2. Maintaining Unrestricted Growth: PO_4 and Yeast Extract

Cells of *Schizochytrium aggregatum* (ATCC 28209) were picked from solid F-1 medium and inoculated into 50ml of FFM medium. (Fuller et al., 1964). This medium contains: seawater, 1000ml; glucose, 1.0g; gelatin hydrolysate, 1.0g; liver extract, 0.01g; yeast extract, 0.1g; PII metals, 5ml; 1ml B-vitamins solution (Goldstein et al., 1969); and 1ml of an antibiotic solution (25g/l streptomycin sulfate and penicillin-G). 1.0ml of the vitamin mix (pH 7.2) contains: thiamine HCl, 200μg; biotin, 0.5μg; cyanocobalamin, 0.05μg; nicotinic acid, 100μg; calcium pantothenate, 100μg; riboflavin, 5.0μg; pyridoxine HCl, 40.0μg; pyridoxamine 2HCl, 20.0μg; p-aminobenzoic acid, 10μg; chlorine HCl, 500μg; inositol, 1.0mg; thymine, 0.8mg; orotic acid, 0.26mg; folinic acid, 0.2μg; and folic acid, 2.5μg. The culture was placed on a rotary shaker (200 rpm) at 27°C. After 3-4 days, 1ml of this culture was transferred to 50ml of each of the following treatments: 1) FFM medium (as control); and 2) FFM

medium with the addition of 250mg/l KH_2PO_4 and 250mg/l yeast extract. These cultures were placed on a rotary shaker (200 rpm) at 27°C for 48 hr. The cells were harvested and the yield of cells quantified. In treatment 1, the final concentration of cells on an ash-free dry weight basis was 616mg/l. In treatment 2, the final concentration of cells was 1675mg/l, demonstrating the enhanced effect of increasing PO_4 and yeast extract concentrations in the culture medium.

Example 3. Maintaining Unrestricted Growth: Substitution of Corn Steep Liquor for Yeast Extract

Cells of *Schizochytrium* sp. S31 (ATCC No. 20888) were picked from solid F-1 medium and placed into 50ml of M-5 medium. This medium consists of (on a per liter basis): yeast extract, 1g; NaCl, 25g; $MgSO_4 \cdot 7H_2O$, 5g; KCl, 1g; $CaCl_2$, 200mg; glucose, 5g; glutamate, 5g; KH_2PO_4, 1g; PII metals, 5ml; A-vitamins solution, 1ml; and antibiotic solution, 1ml. The pH of the solution was adjusted to 7.0 and the solution was filter sterilized. Sterile solutions of corn steep liquor (4g/40ml; pH 7.0) and yeast extract (1g/40ml; pH 7.0) were prepared. To one set of M-5 medium flasks, the following amount of yeast extract solution was added: 1) 2ml; 2) 1.5ml; 3) 1ml; 4) 0.5ml; and 5) 0.25ml. To another set of M-5 medium flasks the yeast extract and corn steep liquor solutions were added at the following levels: 1) 2ml yeast extract; 2) 1.5ml yeast extract and 0.5ml corn steep liquor; 3) 1.0ml yeast extract and 1.0ml corn steep liquor; 4) 0.5ml yeast extract and 1.5ml corn steep liquor; and 5) 2ml corn steep liquor. One ml of the culture in F-1 medium was used

to inoculate each flask. They were placed on a rotary shaker at 27°C for 48 hr. The cells were harvested by centrifugation and the yield of cells (as ash-free dry weight) was determined. The results are shown in Table 1. The results indicate the addition of yeast extract up to 0.8g/l of medium can increase the yield of cells. However, addition of corn steep liquor is even more effective and results in twice the yield of treatments with added yeast extract. This is very advantageous for the economic production of cells as corn steep liquor is much less expensive than yeast extract.

Table 1.

Treatment (Amount Nutrient Supplement Added)	Ash-Free Dry Weight (mg/l)
2.0ml yeast ext.	4000
1.5ml yeast ext.	4420
1.0ml yeast ext.	4300
0.5ml yeast ext.	2780
0.25ml yeast ext.	2700
2.0ml yeast ext.	4420
1.5ml yeast ext. + 0.5ml CSL*	6560
1.0ml yeast ext. + 1.0ml CSL	6640
0.5ml yeast ext. + 1.5ml CSL	7200
2.0ml CSL	7590

*CSL = corn steep liquor

Example 4. Enhanced HUFA Content of Strains Isolated by Method in Example 1 Compared to ATCC Strains (Previously Known Strains)

A battery of 151 newly isolated strains, selected according to the method described in Example 1, were sampled in late exponential phase growth and quantitatively analyzed for HUFA content by gas-liquid chromatography. All strains were grown either in M1 medium or liquid FFM medium, whichever gave highest yield of cells. M1 medium has the same composition as M5 medium, except that the concentrations of glucose and glutamate are 1 g/l. Additionally, five previously isolated *Thraustochytrium* or *Schizochytrium* species were obtained from the American Type Culture Collection, representing all the strains which could be obtained in viable form from the collection. These strains were: *T. aureum* (ATCC No. 28211), *T. aureum* (ATCC No. 34304), *T. roseum* (ATCC No. 28210), *T. straitum* (ATCC No. 34473) and *S. aggregatum* (ATCC No. 28209). The strains all exhibited abbreviated growth in conventional media, and generally showed improved growth in media of the present invention, including M5 medium and FFM medium. The fatty acid production of each of the known strains was measured as described, based upon the improved growth of the strains in media of the invention.

Fatty acid peaks were identified by the use of pure compounds of known structure. Quantitation, in terms of percent by weight of total fatty acids, was carried out by integrating the chromatographic peaks. Compounds identified were: palmitic acid (C16:0), C20:4n-6 and C22:1 (which were

not resolved separately by the system employed), C20:5n-3, C22:5n-6, C22:5n-3, and C22:6n-3. The remainder, usually lower molecular weight fatty acids, were included in the combined category of "other fatty acids." Total omega-3 fatty acids were calculated as the sum of 20:5n-3, 22:5n-3 and 22:6n-3. Total omega-6 fatty acids were calculated as the sum of the 20:4/22:1 peak and the 22:5n-6 peak.

The results are shown in Tables 2-3 and illustrated in Figs. 1-3. From Table 2 it can be seen that large numbers of strains can be isolated by the method of the invention, and that large numbers of strains outperform the previously known strains by several important criteria. For example, 102 strains produced at least 7.8% by weight of total fatty acids C20:5w3, a higher percentage of that fatty acid than any previously known strain. Strains 23B (ATCC No. 20892) and 12B (ATCC No. 20890) are examples of such strains. Thirty (30) strains of the invention produced at least 68% by weight of total fatty acids as omega-3 fatty acids, more than any previously known strain. Strain 23B (ATCC No. 20892) is an example of such strains. Seventy-six (76) strains of the invention yielded not more than 10% by weight of total fatty acids as omega-6 fatty acids, considered undesirable components of the human diet, lower than any previously known strain. Strains 23B (ATCC No. 20892) and 12B (ATCC No. 20890) are examples of such strains. In addition, there are 35 strains of the invention that produce more than 25% by weight of total fatty acids as omega-6 fatty acids, more than any previously known strain. While such

strains may have a more narrow range of uses for dietary purposes, they are useful as feedstock for chemical synthesis of eicosanoids starting from omega-6 fatty acids.

In addition, the data reveal many strains of the invention which produce a high proportion of total omega-3 fatty acids as C22:6n-3. In Table 3, 48 of the strains shown in Table 2 were compared to the previously known strains, showing each of C20:5n-3, C22:5n-3 and C22:6n-3 as percent by weight of total omega-3 content. Fifteen strains had at least 94% by weight of total omega-3 fatty acids as C22:6n-3, more than any previously known strain. Strain S8 (ATCC No. 20889) was an example of such strains. Eighteen strains had at least 28% by weight of total omega-3 fatty acids as C20:5n-3, more than any previously known strain. Strain 12B (ATCC No. 20890) was an example of such strains.

TABLE 2: LIST OF STRAINS AND COMPOSITIONS UNDER STANDARD SCREENING CONDITIONS

PER CENT OF TOTAL FATTY ACIDS							Total	Total	Strain
C16:0	C20:4w6	C20:5w3	C22:5w6	C22:5w3	C22:6w3	Other FA	Omega3	Omega6	
30.4%	2.8%	6.6%	3.2%	0.2%	8.3%	48.5%	15.1%	6.0%	21
22.9%	0.4%	2.3%	15.5%	0.5%	47.0%	11.5%	49.7%	15.9%	ATCC20889
14.9%	6.5%	12.0%	11.8%	0.4%	49.7%	4.7%	62.1%	18.3%	U40-2
40.3%	1.7%	3.8%	8.6%	0.0%	8.2%	37.4%	12.0%	10.2%	21B
20.7%	0.4%	7.8%	0.0%	0.0%	1.1%	70.1%	8.9%	0.4%	BG1
26.0%	5.7%	1.5%	9.7%	0.7%	9.7%	46.7%	11.9%	15.4%	56A
16.4%	1.4%	10.0%	1.9%	2.2%	46.4%	21.0%	58.6%	3.3%	11A-1
23.7%	3.3%	10.5%	1.9%	1.8%	29.9%	28.9%	42.2%	5.2%	4A-1
18.7%	6.9%	9.2%	11.9%	3.2%	25.2%	24.9%	37.5%	18.0%	17B
15.4%	4.2%	7.3%	9.5%	0.9%	51.2%	11.6%	59.3%	13.7%	ATCC20891
22.3%	3.9%	7.6%	23.5%	0.5%	22.1%	20.2%	30.2%	27.4%	S44
14.4%	2.3%	15.0%	18.4%	0.7%	43.8%	5.5%	59.4%	20.7%	U30
22.1%	7.0%	3.1%	12.7%	1.0%	14.9%	38.3%	19.0%	20.5%	S9A
18.1%	2.3%	6.9%	9.1%	0.0%	52.2%	10.6%	59.9%	11.4%	U37-2
15.8%	3.9%	8.8%	11.6%	1.2%	53.3%	5.5%	63.3%	15.5%	S50W
23.7%	3.8%	6.3%	6.9%	0.6%	43.0%	15.6%	50.0%	10.7%	ATCC20891
10.0%	0.0%	0.0%	0.0%	0.0%	0.0%	90.0%	0.0%	0.0%	UX
16.6%	6.3%	11.9%	13.3%	1.7%	43.0%	7.3%	56.6%	19.5%	LW9
17.3%	2.3%	8.4%	11.4%	0.7%	53.6%	6.5%	62.6%	13.6%	C32-2
23.8%	1.2%	6.4%	2.5%	1.9%	34.4%	29.8%	42.6%	3.7%	SA-1
17.1%	5.2%	11.1%	7.6%	2.2%	27.2%	29.6%	40.4%	12.9%	BG1
25.4%	2.2%	9.6%	7.0%	1.1%	46.0%	8.8%	56.7%	9.1%	U3
16.9%	12.0%	6.6%	16.7%	0.4%	25.1%	22.8%	32.1%	28.2%	S5B
26.3%	2.6%	8.6%	2.0%	2.5%	32.4%	25.5%	43.5%	4.6%	10A
19.4%	0.3%	9.8%	0.0%	0.3%	38.4%	31.7%	48.6%	0.3%	32B
16.0%	16.7%	8.6%	18.4%	0.0%	22.5%	17.7%	31.1%	35.1%	56B
18.6%	7.7%	11.4%	3.6%	4.3%	31.7%	22.7%	47.4%	11.2%	SX2
17.8%	4.4%	16.2%	6.4%	3.7%	33.6%	17.8%	53.5%	10.9%	53B
16.8%	2.7%	13.8%	20.5%	1.4%	39.3%	5.5%	54.4%	23.3%	S49
20.8%	8.0%	8.9%	6.4%	1.7%	33.9%	20.3%	44.5%	14.4%	S3
14.8%	0.3%	3.7%	3.9%	0.0%	69.9%	7.4%	73.6%	4.2%	3A-1
28.1%	5.2%	12.7%	3.2%	0.9%	20.9%	29.0%	34.5%	8.4%	15A
20.9%	0.7%	8.5%	1.0%	0.0%	35.0%	33.0%	44.3%	1.7%	9A-1
15.7%	10.2%	8.8%	13.4%	1.5%	23.9%	26.3%	34.3%	23.7%	51B
16.2%	11.2%	7.8%	16.4%	1.5%	20.4%	26.5%	29.7%	27.6%	8A-1
20.5%	5.5%	8.6%	4.8%	2.7%	28.7%	29.2%	40.0%	10.3%	13A-1
16.1%	13.6%	11.1%	16.0%	0.0%	28.4%	14.8%	39.4%	29.6%	24B-2
16.9%	7.3%	16.4%	6.1%	0.0%	40.8%	12.4%	57.2%	13.4%	24B-1
16.2%	0.0%	10.9%	1.0%	0.0%	56.5%	15.5%	67.4%	1.0%	3B
17.0%	0.0%	5.0%	2.3%	0.0%	73.4%	2.3%	78.3%	2.3%	SBG5
20.8%	4.5%	5.8%	3.8%	1.0%	22.7%	41.3%	29.5%	8.4%	16B
19.0%	14.0%	8.3%	18.9%	0.7%	23.9%	15.2%	32.9%	32.9%	6A-1
18.0%	0.3%	10.1%	0.0%	0.0%	48.9%	22.7%	59.0%	0.3%	33B

PER CENT OF TOTAL FATTY ACIDS							Total	Total	Strain
C16:0	C20:4w6	C20:5w3	C22:5w6	C22:5w3	C22:6w3	Other FA	Omega3	Omega6	
16.7%	5.5%	14.8%	8.5%	1.7%	31.8%	21.0%	48.3%	13.9%	B40
15.0%	1.0%	11.7%	2.1%	0.9%	62.3%	6.9%	74.9%	3.1%	28A
17.8%	10.5%	8.1%	20.5%	0.0%	22.1%	12.9%	30.2%	39.0%	43B
16.9%	0.0%	3.4%	2.7%	0.0%	61.2%	15.8%	64.6%	2.7%	1A-1
15.6%	2.7%	11.4%	10.9%	0.8%	53.7%	4.9%	65.9%	13.6%	U41-2
16.5%	0.7%	3.9%	3.9%	0.0%	68.4%	6.7%	72.2%	4.6%	56B
14.4%	0.9%	10.9%	2.5%	1.0%	66.4%	3.8%	78.3%	3.4%	46A
17.6%	0.0%	2.4%	3.3%	0.0%	66.3%	10.4%	68.7%	3.3%	15A-1
25.0%	0.0%	3.3%	0.0%	1.4%	53.2%	17.1%	57.9%	0.0%	13A
16.1%	13.4%	9.3%	13.6%	0.0%	32.3%	15.3%	41.6%	27.0%	32B
16.5%	9.1%	13.2%	6.7%	0.0%	38.9%	15.6%	52.1%	15.9%	43B
16.1%	12.4%	12.0%	15.7%	0.0%	30.5%	12.5%	43.3%	28.1%	17B
13.8%	0.0%	11.5%	2.8%	0.0%	67.0%	4.1%	78.6%	3.6%	27A
17.5%	18.6%	9.0%	19.5%	0.0%	21.7%	13.7%	30.7%	38.1%	46B
21.4%	1.4%	18.9%	0.0%	5.0%	43.5%	9.9%	67.3%	1.4%	ATCC20890
17.7%	0.0%	0.6%	4.4%	0.0%	68.2%	9.1%	68.8%	4.4%	5A
17.6%	16.0%	9.6%	18.8%	0.0%	25.6%	12.4%	35.2%	34.8%	28B-2
14.0%	0.9%	13.2%	1.6%	0.0%	64.7%	5.5%	77.9%	2.6%	27B
19.5%	2.9%	16.6%	1.1%	1.6%	30.2%	28.1%	48.5%	4.0%	49B
17.2%	0.7%	6.8%	2.7%	0.0%	63.0%	9.6%	69.8%	3.4%	18B
14.4%	3.5%	13.5%	26.0%	1.0%	37.2%	4.4%	51.6%	29.5%	S49-2
16.1%	2.2%	15.7%	21.6%	0.0%	36.7%	7.8%	52.4%	23.7%	20B
17.3%	4.7%	14.3%	7.2%	2.9%	30.2%	23.5%	47.3%	11.9%	8B
11.5%	3.3%	11.3%	6.5%	1.1%	59.9%	6.5%	72.2%	9.8%	13B
16.6%	0.7%	10.7%	1.6%	0.0%	59.7%	10.8%	70.4%	2.2%	26A
16.1%	3.3%	13.5%	23.8%	0.0%	38.7%	4.7%	52.2%	27.1%	S42
15.6%	0.6%	12.1%	0.0%	0.0%	60.2%	11.5%	72.3%	0.6%	35B
19.5%	0.0%	1.4%	3.4%	0.0%	66.6%	9.1%	68.0%	3.4%	42A
18.9%	3.5%	12.7%	25.0%	0.0%	35.0%	5.0%	47.6%	28.5%	40A
25.2%	3.3%	9.3%	21.8%	0.0%	30.3%	10.1%	39.6%	25.1%	S50C
17.6%	11.1%	13.2%	14.1%	1.3%	28.7%	14.0%	43.2%	25.2%	59A
19.9%	0.0%	5.5%	1.9%	0.0%	66.8%	6.0%	72.3%	1.9%	SBG9
15.4%	3.1%	13.2%	26.1%	0.0%	35.8%	6.5%	49.1%	29.1%	21B
18.9%	0.7%	11.6%	0.0%	0.0%	59.1%	9.7%	70.7%	0.7%	2B
14.1%	1.1%	12.4%	2.0%	0.0%	65.2%	5.2%	77.6%	3.1%	1B
22.2%	16.2%	6.3%	17.7%	0.0%	18.1%	19.5%	24.4%	33.8%	55B
16.0%	1.0%	4.5%	0.0%	0.0%	69.5%	9.0%	74.0%	1.0%	3A
17.0%	4.3%	12.4%	29.8%	0.0%	34.0%	2.5%	46.4%	34.1%	9B
15.4%	4.3%	8.7%	13.2%	0.0%	53.2%	5.1%	62.0%	17.5%	U24
14.2%	3.1%	12.0%	20.0%	1.1%	35.2%	14.3%	48.3%	23.2%	U28
16.8%	14.6%	10.1%	16.0%	0.6%	27.7%	14.0%	38.5%	30.7%	28B-1
23.2%	1.9%	8.3%	1.1%	2.3%	22.7%	40.4%	33.3%	3.0%	44B
24.6%	15.8%	8.7%	16.0%	0.0%	15.3%	19.6%	24.0%	31.8%	54B
15.5%	0.0%	1.3%	2.9%	0.0%	72.7%	7.6%	74.0%	2.9%	55A
18.4%	1.0%	5.0%	3.0%	0.0%	66.2%	6.4%	71.3%	3.9%	49A
18.6%	15.3%	9.4%	18.0%	0.0%	27.3%	11.4%	36.7%	33.3%	51A
23.5%	13.1%	7.3%	17.9%	0.0%	26.7%	11.4%	34.0%	31.0%	14A-1
13.3%	1.1%	14.5%	0.9%	0.0%	64.6%	5.6%	79.1%	2.0%	25B
22.9%	2.4%	10.3%	21.5%	0.0%	26.5%	16.4%	36.9%	23.9%	41A
16.8%	1.0%	9.7%	2.7%	0.0%	58.3%	11.5%	68.0%	3.7%	24A
0.4%	8.5%	14.1%	10.2%	2.1%	27.6%	37.0%	43.8%	18.8%	61A

PER CENT OF TOTAL FATTY ACIDS							Total	Total	Strain
C16:0	C20:4w6	C20:5w3	C22:5w6	C22:5w3	C22:6w3	Other FA	Omega3	Omega6	
30.5%	0.0%	7.1%	0.0%	0.0%	0.6%	61.8%	7.7%	0.0%	BRHG
10.2%	14.9%	8.3%	10.7%	0.0%	24.4%	15.5%	32.7%	33.6%	17A
17.4%	2.0%	9.3%	2.0%	0.0%	55.7%	12.7%	65.0%	4.9%	60A
14.1%	0.8%	13.0%	1.2%	0.0%	67.8%	3.1%	80.8%	2.0%	26B
17.8%	5.0%	6.9%	15.0%	1.5%	47.4%	6.4%	55.8%	20.0%	AICC20808
16.0%	0.0%	1.8%	2.0%	0.0%	70.8%	9.4%	72.6%	2.0%	2A
24.6%	0.0%	4.0%	0.0%	0.0%	49.4%	22.0%	53.4%	0.0%	44A
17.4%	1.8%	0.0%	2.9%	0.0%	55.3%	23.3%	55.3%	4.6%	14A
23.3%	1.3%	4.6%	0.0%	0.0%	12.6%	58.4%	17.3%	1.3%	41B
19.3%	0.0%	1.1%	3.8%	0.0%	66.6%	9.1%	67.8%	3.8%	66A
10.6%	15.6%	8.3%	17.1%	1.1%	24.6%	14.8%	33.9%	32.7%	11A
19.6%	5.1%	10.1%	27.7%	0.0%	27.5%	10.6%	37.5%	32.3%	2%
15.7%	2.4%	14.0%	25.7%	0.0%	36.7%	5.4%	50.8%	28.1%	33A
14.6%	1.5%	13.5%	0.0%	0.0%	66.0%	4.3%	79.5%	1.5%	AICC20892

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PER CENT OF TOTAL FATTY ACIDS							Total	Total	Strain
C16:0	C20:4w6	C20:5w3	C22:5w6	C22:5w3	C22:6w3	Other FA	Omega3	Omega6	
15.7%	3.9%	3.7%	8.1%	0.0%	55.1%	13.5%	50.8%	12.0%	AICC34304
20.2%	1.6%	6.9%	11.4%	0.0%	17.8%	34.1%	24.7%	12.9%	AICC24473
15.2%	2.9%	7.7%	9.8%	0.6%	54.6%	9.2%	62.9%	12.7%	AICC20211
23.2%	10.7%	4.3%	12.6%	1.5%	20.6%	27.0%	26.4%	23.4%	AICC20209
13.2%	6.3%	6.9%	4.3%	0.0%	60.1%	9.1%	67.0%	10.6%	AICC20210

TABLE 3: COMPOSITION OF OMEGA 3 FATTY ACID FRACTION

EPA C20:5w3	DPA C22:5w3	DHA C22:6w3	Strain
44.0%	1.1%	54.9%	21
4.6%	0.9%	94.5%	ATCC20889
19.3%	0.7%	80.0%	U40-2
31.9%	0.0%	68.1%	21B
87.9%	0.0%	12.1%	BRBG1
12.5%	6.1%	81.5%	56A
17.0%	3.7%	79.3%	11A-1
24.9%	4.3%	70.8%	4A-1
24.4%	8.4%	67.2%	17B
12.2%	1.5%	86.3%	ATCC20891
25.1%	1.7%	73.2%	S44
25.2%	1.1%	73.7%	U30
16.2%	5.4%	78.4%	59A
11.5%	1.4%	87.1%	U37-2
14.0%	1.9%	84.2%	S50W
12.7%	1.3%	86.0%	ATCC20891
---	---	---	UX
21.0%	2.9%	76.1%	LWN9
13.4%	1.0%	85.6%	C32-2
15.0%	4.3%	80.7%	5A-1
27.4%	5.4%	67.2%	BRBG1
17.0%	1.9%	81.1%	U3
20.5%	1.3%	78.2%	55B
19.8%	5.8%	74.4%	18A
20.1%	0.7%	79.2%	32B
27.8%	0.0%	72.2%	56B
24.1%	9.1%	66.9%	SX2
30.3%	6.9%	62.8%	53B
25.3%	2.5%	72.2%	S49
19.9%	3.8%	76.3%	S3
5.0%	0.0%	95.0%	3A-1
36.9%	2.6%	60.5%	15A
19.3%	0.0%	80.7%	9A-1
25.8%	4.4%	69.8%	51B
26.3%	5.0%	68.7%	8A-1
21.6%	6.7%	71.7%	13A-1
28.0%	0.0%	72.0%	24B-2
28.7%	0.0%	71.3%	24B-1
16.2%	0.0%	83.8%	3B
6.3%	0.0%	93.7%	SBG5
19.7%	3.3%	77.0%	16B
25.2%	2.1%	72.6%	6A-1
17.1%	0.0%	82.9%	33B
30.5%	3.6%	65.9%	B40
15.6%	1.2%	83.1%	20A

EPA C20:5w3	DPA C22:5w3	DHA C22:6w3	Strain
26.8%	0.0%	73.2%	43B
5.2%	0.0%	94.8%	1A-1
17.4%	1.2%	81.5%	U41-2
5.4%	0.0%	94.6%	56B
13.9%	1.3%	84.8%	46A
3.5%	0.0%	96.5%	15A-1
5.8%	2.4%	91.8%	13A
22.3%	0.0%	77.7%	37B
25.4%	0.0%	74.6%	43B
27.7%	1.9%	70.3%	17B
14.7%	0.0%	85.3%	27A
29.2%	0.0%	70.8%	46B
28.0%	7.5%	64.5%	ATCC20890
0.9%	0.0%	99.1%	5A
27.3%	0.0%	72.7%	28B-2
16.9%	0.0%	83.1%	27B
34.3%	3.4%	62.3%	49B
9.7%	0.0%	90.3%	10B
26.1%	1.9%	71.9%	S49-2
29.9%	0.0%	70.1%	20B
30.1%	6.2%	63.7%	8B
15.6%	1.5%	82.9%	13B
15.2%	0.0%	84.8%	26A
25.9%	0.0%	74.1%	S42
16.7%	0.0%	83.3%	35B
2.1%	0.0%	97.9%	42A
26.6%	0.0%	73.4%	40A
23.4%	0.0%	76.6%	S50C
30.6%	2.9%	66.4%	59A
7.6%	0.0%	92.4%	S8G9
27.0%	0.0%	73.0%	21B
16.4%	0.0%	83.6%	2B
15.9%	0.0%	84.1%	1B
25.9%	0.0%	74.1%	55B
6.0%	0.0%	94.0%	3A
26.7%	0.0%	73.3%	9B
14.1%	0.0%	85.9%	U24
24.9%	2.2%	72.9%	U20
26.4%	1.5%	72.1%	28B-1
24.8%	6.9%	68.3%	44B
36.4%	0.0%	63.6%	54B
1.8%	0.0%	98.2%	55A
7.1%	0.0%	92.9%	49A
25.6%	0.0%	74.4%	51A
21.5%	0.0%	78.5%	14A-1
18.4%	0.0%	81.6%	25B
28.1%	0.0%	71.9%	41A
14.3%	0.0%	85.7%	24A
32.3%	4.8%	63.0%	61A
91.6%	0.0%	8.4%	BRDG

EPA C20:5w3	DPA C22:5w3	DHA C22:6w3	Strain
25.5%	0.0%	74.5%	17A
14.4%	0.0%	85.6%	60A
16.1%	0.0%	83.9%	26B
12.4%	2.7%	84.9%	ATCC20808
2.5%	0.0%	97.5%	2A
7.5%	0.0%	92.5%	44A
0.0%	0.0%	100.0%	14A
26.7%	0.0%	73.3%	41B
1.7%	0.0%	98.3%	66A
24.5%	3.1%	72.4%	11A
26.8%	0.0%	73.2%	2%
27.6%	0.0%	72.4%	33A
17.0%	0.0%	83.0%	ATCC20092

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EPA C20:5w3	DPA C22:5w3	DHA C22:6w3	Strain
6.4%	0.0%	93.6%	ATCC34304
27.9%	0.0%	72.1%	ATCC24473
12.2%	1.0%	86.8%	ATCC20211
16.4%	5.6%	78.1%	ATCC20209
10.3%	0.0%	89.7%	ATCC20210

Fig. 1 illustrates the set of strains, isolated by the method in Example 1, that have more than 67% omega-3 fatty acids (as % of total fatty acids) and less than 10.6% omega-6 fatty acids (as % of total fatty acids). All of the previously known strains had less than 67% omega-3 fatty acids (as % of total fatty acids) and greater than 10.6% omega-6 (as % of total fatty acids).

Fig. 2 illustrates the set of strains, isolated by the method in Example 1, that have more than 67% omega-3 fatty acids (as % of total fatty acids) and greater than 7.5% C20:5n-3 (as % of total fatty acids). All of the previously known strains had less than 67% omega-3 fatty acids (as % of total fatty acids) and less than 7.8% C20:5n-3 (as % of total fatty acids).

Example 5. Enhanced Growth Rates of Strains Isolated by Method in Example 1 Compared to ATCC Strains (Previously Known Strains)

Cells of *Schizochytrium* sp. S31 (ATCC No. 20888), *Schizochytrium* sp. S8 (ATCC No. 20889), *Thraustochytrium* sp. S42, *Thraustochytrium* sp. U42-2, *Thraustochytrium* sp. S42 and U30, (all isolated by the method of Example 1) and *Thraustochytrium aureum* (ATCC #28211) and *Schizochytrium aggregatum* (ATCC #28209) (previously known strains) were picked from solid F-1 medium and placed into 50ml of M-5 medium. The pH of the solution was adjusted to 7.0 and the solution was filter sterilized. After three days of growth on an orbital shaker (200 rpm, 27°C), 1-2ml of each culture was transferred to another flask of M-5 medium and placed

on the shaker for 2 days. The cultures (1-2ml) were then transferred to another flask of M-5 medium and placed on the shaker for 1 day. This process ensured that all cultures were in the exponential phase of growth. These later cultures were then used to inoculate two 250ml flasks of M-5 medium for each strain. These flasks were than placed on shakers at 25°C and 30°C, and changes in their optical density were monitored on a Beckman DB-G spectrophotometer (660nm, 1cm path length). Optical density readings were taken at the following times: 0, 6, 10, 14, 17.25, 20.25 and 22.75 hours. Exponential growth rates (doublings/day) were then calculated from the optical density data by the method of Sorokin (1973). The results are presented in Table 4 and illustrated (normalized to the growth of strain U30 at 25°C) in Fig. 4. The data indicate that the strains isolated by the method in Example 1 have much higher growth rates than the previously known ATCC strains at both 25°C and 30°C, even under the optimized phosphate levels essential for continuous growth. Strains of Thraustochytriales isolated from cold Antarctic waters have not been shown to grow at 30°C.

Table 4. Exponential Growth Rate (doublings/day)

Strain	25°C	30°C
S31* (ATCC No. 20888)	8.5	9.4
U40-2*	5.8	6.0
S8* (ATCC No. 20889)	7.1	8.8
S42*	6.6	8.3
U30*	5.5	7.3
28209**	4.6	5.0
28210**	3.5	4.5
28211**	4.2	5.7
34304**	2.7	3.7
24473**	4.6	5.3

* strain isolated by method in Example 1

** previously known ATCC strain

Example 6. Enhanced Production Characteristics (Growth and Lipid Induction) of Strains Isolated by Method in Example 1 Compared to ATCC Strains (Prior Art Strains)

Cells of *Schizochytrium* sp. S31 (ATCC No. 20888), *Schizochytrium* sp. S8 (ATCC No. 20889) (both isolated by the method of Example 1) and *Thraustochytrium aureum* (ATCC #28211) and *Schizochytrium aggregatum* (ATCC #28209) (prior art strains) were picked from solid F-1 medium and placed into 50ml of M-5 medium (see Example 3). The pH of the solution was adjusted to 7.0 and the solution was filter sterilized. After three days of growth on an orbital shaker (200 rpm, 27°C), 1-2ml of each culture was transferred to another flask of M-5 medium and placed on the shaker for 2

days. The ash-free dry weights for each of these cultures were then quickly determined and then 3.29mg of each culture was pipetted into two 250ml erlenmeyer flasks containing 50ml of M-5 medium. These flasks were placed on a rotary shaker (200 rpm, 27°C). After 24 hours 20ml portions of each culture were then centrifuged, the supernatants discarded, and the cells transferred to 250ml erlenmeyer flasks containing 50 ml of M-5 medium without any glutamate (N-source). The flasks were placed back on the shaker, and after another 12 hours they were sampled to determine ash-free dry weights and quantify fatty acid contents by the method of Lepage and Roy (1984). The results are illustrated (normalized to the yields of ATCC No. 28211, previously known strain) in Fig. 5. The results indicate that the strains isolated by the method of Example 1 produced 2-3 times as much ash-free dry weight in the same period of time, under a combination of exponential growth and nitrogen limitation (for lipid induction) as the prior art ATCC strains. In addition, higher yields of total fatty acids and omega-3 fatty acids were obtained from strains of the present invention with strains S31 (ATCC No. 20888) producing 3-4 times as much omega-3 fatty acids as the prior art ATCC strains.

Example 7. Enhanced Lower Salinity Tolerance and Fatty Acid Production by Strains Isolated by Method in Example 1

Strains of 4 species of Thraustochytrids, *Schizochytrium* sp. S31 (ATCC No. 20888) and *Thraustochytrium* sp. U42-2 (ATCC No. 20891) (both isolated and screened by the method of

Example 1), and *S. aggregatum* (ATCC 28209) and *T. aureum* (ATCC 28210) (obtained from the American Type Culture Collection) were picked from solid F-1 medium and incubated for 3-4 days at 27°C on a rotary shaker (200 rpm). A range of differing salinity medium was prepared by making the following dilutions of M medium salts (NaCl, 25g/l; $MgSO_4 \cdot 7H_2O$, 5g/l; KCl, 1g/l; $CaCl_2$, 200mg/l: 1) 100% (w/v M medium salts; 2) 80% (v/v) M medium, 20% (v/v) distilled water; 3) 60% (v/v) M medium, 40% (v/v) distilled water; 4) 40% (v/v) M medium, 60% (v/v) distilled water; 5) 20% (v/v) M medium, 80% distilled water; 6) 15% (v/v) M medium, 85% (v/v) distilled water; 7) 10% (v/v) M medium, 90% (v/v) distilled water; 8) 7% (v/v) M medium, 93% (v/v) distilled water; 9) 3% (v/v) M medium, 97% (v/v) distilled water; 10) 1.5% (v/v) M medium, 98.5% (v/v) distilled water. The following nutrients were added to the treatments (per liter): glucose, 5g; glutamate, 5g; yeast ext., 1g; $(NH_4)_2SO_4$, 200 mg; $NaHCO_3$, 200 mg; PII metals, 5ml; A-vitamins solution, 1ml; and antibiotics solution, 2ml. Fifty ml of each of these treatments were inoculated with 1ml of the cells growing in the F-1 medium. These cultures were placed on an orbital shaker (200 rpm) and maintained at 27°C for 48 hr. The cells were harvested by centrifugation and total fatty acids determined by gas chromatography. The results are illustrated in Fig. 6. *Thraustochytrium* sp. U42-2 (ATCC No. 20891) isolated by the method of Example 1 can yield almost twice the amount of fatty acids produced by *T. aureum* (ATCC 28211) and over 8 times the amount of fatty acids

produced by *S. aggregatum* (ATCC 28209). Additionally, U42-2 appears to have a wider salinity tolerance at the upper end of the salinity range evaluated. *Schizochytrium* sp. S31 (ATCC No. 20888), also isolated by the method in Example 1, exhibited both a high fatty acid yield (2.5 to 10 times that of the previously known ATCC strains) and a much wider range of salinity tolerance than the ATCC strains. Additionally, *Schizochytrium* sp. S31 (ATCC No. 20888) grows best at very low salinities. This property provides a strong economic advantage when considering commercial production, both because of the corrosive effects of saline waters on metal reactors, and because of problems associated with the disposal of saline waters.

Example 8. Cultivation/Low Salinity

Fifty ml of M/10-5 culture media in a 250ml erlenmeyer flask was inoculated with a colony of *Schizochytrium* sp. S31 (ATCC No. 20888) picked from an agar slant. The M/10-5 media contains: 1000ml deionized water, 2.5g NaCl, 0.5g $MgSO_4 \cdot 7H_2O$, 0.1g KCl, 0.02g $CaCl_2$, 1.0g KH_2PO_4, 1.0g yeast extract, 5.0g glucose, 5.0g glutamic acids, 0.2g $NaHCO_3$, 5ml PII trace metals, 2ml vitamin mix, and 2ml antibiotic mix. The culture was incubated at 30°C on a rotary shaker (200 rpm). After 2 days the culture was at a moderate density and actively growing. 20ml of this actively growing culture was used to inoculate a 2 liter fermenter containing 1700ml of the same culture media except the concentration of the glucose and glutamate had been increased to 40g/l (M/10-40 media). The

fermenter was maintained at 30°C, with aeration at 1 vol/vol/min, and mixing at 300 rpm. After 48 hr, the concentration of cells in the fermenter was 21.7g/l. The cells were harvested by centrifugation, lyophilized, and stored under N_2.

The total fatty acid content and omega-3 fatty acid content was determined by gas chromatography. The total fatty acid content of the final product was 39.0% ash-free dry weight. The omega-3 HUFA content (C20:5n-3, C22:5n-3 and C22:6n-3) of the microbial product was 25.6% of the total fatty acid content. The ash content of the sample was 7.0%.

Example 9. Diversity of Fatty Acid Content

Growth and gas chromatographic analysis of fatty acid production by various strains as described in Example 4 revealed differences in fatty acid diversity. Strains of the present invention synthesized fewer different fatty acids than previously available strains. Lower diversity of fatty acids is advantageous in fatty acid purification since there are fewer impurities to be separated. For food supplement purposes, fewer different fatty acids is advantageous because the likelihood of ingesting unwanted fatty acids is reduced. Table 5 shows the number of different HUFAs present, at concentrations greater than 1% by weight of total fatty acids for previously known strains, designated by ATCC number and various strains of the present invention.

Table 5.

Strain	No. of Different Fatty Acids at 1% or Greater % of Total Fatty Acids
34304**	8
28211**	8
24473**	10
28209**	13
28210**	8
S31*	5
S8*	6
79B*	6

* strain isolated by the method in Example 1

** previously known ATCC strain

Example 10. Recovery

Fifty ml of M5 culture media in a 250 ml erlenmeyer flask was inoculated with a colony of *Schizochytrium* sp. S31 (ATCC No. 20888) picked from an agar slant. The culture was incubated at 30°C on a rotary shaker (200 rpm). After 2 days the culture was at a moderate density and actively growing. 20ml of this actively growing culture was used to inoculate a 1 liter fermenter containing 1000ml of the same culture media except the concentration of the glucose and glutamate had been increased to 40g/l (M20 media). The fermenter was maintained at 30°C and pH 7.4, with aeration at 1 vol/min, and mixing at 400 rpm. After 48 hr, the concentration of the cells in the fermenter was 18.5g/l. Aeration and mixing in the fermenter was turned off. Within 2-4 minutes, the

cells flocculated and settled in the bottom 250 ml of the fermenter. This concentrated zone of cells had a cell concentration of 72g/l. This zone of cells can be siphoned from the fermenter, and: (1) transferred to another reactor for a period of nitrogen limitation (e.g., combining the highly concentrated production of several fermenters); or (2) harvested directly by centrifugation or filtration. By preconcentrating the cells in this manner, 60-80% less water has to be processed to recover the cells.

Example 11. Utilization of a Variety of Carbon and Nitrogen Sources

Fifty ml of M5 culture media in a 250ml erlenmeyer flask was inoculated with a colony of *Schizochytrium* sp. S31 (ATCC No. 20888) or *Thraustochytrium* sp. U42-2 (ATCC No. 20891) picked from an agar slant. The M5 media was described in Example 3 except for the addition of 2ml vitamin mix, and 2ml antibiotic mix. The culture was incubated at 30°C on a rotary shaker (200 rpm). After 2 days the culture was at a moderate density and actively growing. This culture was used to inoculate flasks of M5 media with one of the following substituted for the glucose (at 5g/l): dextrin, sorbitol, fructose, lactose, maltose, sucrose, corn starch, wheat starch, potato starch, ground corn; or one of the following substituted for the glutamate (at 5g/l): gelysate, peptone, tryptone, casein, corn steep liquor, urea, nitrate, ammonium, whey, or corn gluten meal. The cultures were incubated for 48 hours on a rotary shaker (200 rpm, 27°C).

The relative culture densities, representing growth on the different organic substrates, are illustrated in Tables 6-7.

Table 6. Utilization of Nitrogen Sources

N-Source	Strains	
	Thraustochytrium sp. U42-2 ATCC No. 20891	*Schizochytrium* sp. S31 ATCC No. 20888
glutamate	+++	+++
gelysate	+++	+++
peptone	++	++
tryptone	++	++
casein	++	++
corn steep liquor	+++	+++
urea	+	++
nitrate	++	+++
ammonium	+	+++
whey	+++	+++
corn gluten meal	+++	+++

+++ = high growth
++ = medium growth
+ = low growth
0 = no growth

Table 7. Utilization of Organic Carbon Sources

C-Source	Strains	
	Thraustochytrium sp. U42-2 ATCC No. 20891	*Schizochytrium* sp. S31 ATCC No. 20888
glucose	+++	+++
dextrin	+++	+++
sorbitol	+	+
fructose	+	+++
lactose	+	+
maltose	+++	+
sucrose	+	+
corn starch	+++	+
wheat starch	+++	+
potato starch	+++	+
ground corn	+++	0

+++ = high growth
++ = medium growth
+ = low growth
0 = no growth

Example 12. Feeding of Thraustochytrid-Based Feed Supplement to Brine Shrimp to Increase Their Omega-3 HUFA Content

Cellular biomass of *Thraustochytrium* sp. 12B (ATCC 20890) was produced in shake flasks in M-5 medium (see Example 3) at 25°C. Cellular biomass of *Thraustochytrium* sp. S31 (ATCC 20888) was produced in shake flasks in M/10-5 medium (see Example 8) at 27°C. The cells of each strain were harvested by centrifugation. The pellet was washed once with distilled water and recentrifuged to produce a 50% solids paste. The resulting paste was resuspended in sea

water and then added to an adult brine shrimp culture as a feed supplement. The brine shrimp had previously been reared on agricultural waste products and as a result their omega-3 HUFA content was very low, only 1.3 - 2.3% of total fatty acids (wild-caught brine shrimp have an average omega-3 HUFA content of 6 - 8% total fatty acids). The brine shrimp (2 - 3/mL) were held in a 1 liter beaker filled with sea water and an airstone was utilized to aerate and mix the culture. After addition of the feed supplement, samples of the brine shrimp were periodically harvested, washed, and their fatty acid content determined by gas chromatography. The results are illustrated in Figs. 7 and 8. When fed the thraustochytrid-based feed supplement as a finishing feed, the omega-3 content of the brine shrimp can be raised to that of wild-type brine shrimp within 5 hours if fed strain 12B or within 11 hours when fed strain S31. The omega-3 HUFA content of the brine shrimp can be greatly enhanced over that of the wild type if fed these feed supplements for up to 24 hours. Additionally, these feed supplements greatly increase the DHA content of the brine shrimp, which is generally only reported in trace levels in wild-caught brine shrimp.

Example 13. Use of Sodium Sulfate in Culture Medium

This example illustrates that omega-3 production and total fatty acid content is not harmed and can be the same or better when using sodium sulfate instead of sodium chloride as the sodium salt in a fermentation medium.

Schizochytrium ATCC No. 20888 was grown in medium, pH 7.0, containing 2.36 grams of sodium per liter of medium, 1.5-3.0 grams of a nitrogen source per liter of medium, and 3.0 grams of glucose per liter of medium. The cells were incubated at 28°C, at 200 rotations per minute, for 48 hours. The results are shown in Table 8.

Table 8. Effect of Sodium Sulfate Compared With Sodium Chloride on Fatty Acid Content

A) Na salt = sodium chloride; N source = sodium glutamate

N source (g/L)	omega-3 (% dwt)	total fatty acid (% dwt)	biomass yield (g/L)
3.0	6.0	11.2	1.74
2.5	5.8	10.8	1.71
2.0	5.8	11.0	1.65
1.5	7.5	20.3	1.39

B) Na salt = sodium chloride; N source = peptone

N source (g/L)	omega-3 (% dwt)	total fatty acid (% dwt)	biomass yield (g/L)
3.0	7.9	21.9	1.34
2.5	9.4	27.4	1.21
2.0	6.7	28.9	1.18
1.5	11.1	42.1	1.16

C) Na salt = sodium sulfate; N source = sodium glutamate

N source (g/L)	omega-3 (% dwt)	total fatty acid (% dwt)	biomass yield (g/L)
3.0	9.3	31.9	1.34
2.5	10.1	38.6	1.35
2.0	10.1	41.4	1.30
1.5	9.5	43.6	1.26

As seen in Table 8, omega-3 and total fatty acid production when using sodium sulfate is comparable to or better than when using sodium chloride as a sodium salt.

Example 14. Production of *Schizochytrium* in Low Salinity Culture Medium

This Example illustrates the fermentation of *Schizochytrium* in a low salinity culture medium while maintaining high biomass yields and high omega-3 and fatty acid production.

Schizochytrium ATCC No. 20888 was grown in medium, containing 3.33g/l of peptone as a nitrogen source, 5.0g/l of glucose as a carbon source, with varying sodium concentrations. The cells were fermented at 30°C with an inoculum of about 40mg/L dwt for a period of 48 hours. The sodium was supplied as sodium chloride. The results of this run are shown in Table 9.

Table 9. Production of *Schizochytrium* in Low Salinity Culture Medium

Na conc. g/L	Cl conc. g/L	Biomass Yield g/L	Fatty acids % dwt	omega-3 % dwt	final glucose g/L
4.88	7.12	1.76±0.60	35.4±1.0	10.2±0.6	0.00
3.90	5.70	1.72±0.67	37.0±0.7	11.1±0.3	0.15
2.93	4.27	1.70±0.42	43.0±0.2	12.1±0.1	0.22
1.95	2.85	1.66±0.57	29.8±0.7	9.3±0.1	1.55
0.98	1.42	0.40±0.61	10.6±2.4	4.0±1.0	4.31

As can be seen from the results in Table 9, high biomass yields and production of omega-3 fatty acids and total fatty acids can be achieved at sodium concentrations of greater than about 1.0 g/l.

Example 15. Cultivation of *Schizochytrium* in Medium with Low Chloride Content

This Example illustrates the fermentation of microflora of the present invention at minimal chloride concentrations while achieving high biomass yields based on starting sugar concentration.

Schizochytrium ATCC No. 20888 was cultured in shake flasks at 200 rpm and 28°C in 50ml aliquots of the following medium. 1000ml deionized water; 1.2g Mg $SO_4.7H_2O$; 0.067g $CaCO_3$; 3.0g glucose; 3.0g monosodium glutamate; 0.2g KH_2PO_4; 0.4g yeast extract; 5.0ml PII metals, 1.0 vitamin mix; and 0.1g each of penicillin-G and streptomycin sulfate. The chloride concentration was varied by adding differing amounts of KCl to each treatment. The potassium concentration in all of the treatments was held constant by additions of potassium citrate. Sodium concentration was either 2.37g/l or 4.0 g/l through addition of sodium sulfate. The results of these fermentations are shown below in Table 10.

Table 10. Fermentation of *Schizochytrium* at Minimal Chloride Concentrations

Chloride conc. (mg/L)	Na 2.37 g/L Biomass Yield (mg/L)	Na 4.0 g/L Biomass Yield (mg/L)
0.1	198 ± 21	158 ± 48
7.1	545 ± 120	394 ± 151
15.1	975 ± 21	758 ± 163
30.1	1140 ± 99	930 ± 64
59.1	1713 ± 18	1650 ± 14
119.1	1863 ± 53	1663 ± 46
238.1	1913 ± 11	1643 ± 39

As can be seen from the results shown in Table 10, high yields of biomass per sugar can be achieved at low chloride concentrations. For example, at a chloride concentration of greater than 59.1mg/L, yields of greater than 50% are achieved.

Example 16. Variation of Sodium Sulfate Concentration at Low Chloride Concentrations

This Example illustrates the effect of varying sodium sulfate concentration in a fermentation at low chloride concentration.

Schizochytrium ATC 20888 was cultured in shake flasks at 200 rpm and 28°C in 50 ml aliquots of the following medium: 1000ml deionized water; 1.2g $MgSO_4.7H_2O$; 0.125g KCl; 0.067g $CaCO_3$; 3.0g glucose; 3.0g monosodium glutamate; 0.2g KH_2PO_4; 0.4g yeast extract; 5.0ml PII metals; 1.0ml vitamin mix; and 0.1g each of penicillin-G and streptomycin sulfate. The sodium sulfate concentration was varied in the treatments from 3.0g/l to 30.2g/l. The results of the fermentation runs are shown below in Table 11.

Table 11. Variation of Sodium Sulfate Concentration at Low Chloride Content

Sodium Sulfate (g/l)	Biomass yield (g/l)
3.0	0.78
6.0	1.13
9.1	1.72
12.1	1.88
15.1	1.89
22.7	1.91
30.2	1.63

The results shown in Table 11, illustrate that at a low chloride concentration of about 59g/l, high biomass yields from glucose of greater than 50% can be obtained by selection of an appropriate sodium sulfate concentration.

What is claimed is:

1. A process for growing *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, comprising growing said *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, in a culture medium containing a non-chloride sodium salt.

2. The process of Claim 1, wherein said sodium salt comprises sodium sulfate.

3. The process of Claim 2, wherein the concentration of said sodium sulfate, expressed as grams of sodium per liter of culture medium, is greater than about 1.0.

4. The process of Claim 2, wherein the concentration of said sodium sulfate, expressed as grams of sodium per liter of culture medium, is between about 1.0 and about 50.0.

5. The process of Claim 2, wherein the concentration of said sodium sulfate, expressed as grams of sodium per liter of culture medium, is between about 2.0 and about 25.0.

6. The process of Claim 1, wherein said *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, have all of the identifying characteristics of an organism selected from the group consisting of ATCC Nos. 20888 and 20889, and mutants thereof, wherein said mutants have an omega-3 HUFA content of at least about 0.5% dry weight.

7. The process of Claim 1, wherein of said *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, have a sterol content of at least about 0.1% afdw.

8. The process of Claim 7, wherein *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, have a cholesterol content of at least about 15% of the total sterol content.

9. The process of Claim 1, wherein said *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, have a cell aggregate size less than about 150 microns in diameter.

10. The process of Claim 1, wherein said *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, have a cell aggregate size less than about 100 microns in diameter.

11. The process of Claim 1, wherein said *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, have a cell aggregate size less than about 50 microns in diameter.

12. The process of Claim 1, wherein said culture medium has a chloride concentration of less than about 3 grams of chloride per liter of culture medium.

13. The process of Claim 1, wherein said culture medium has a chloride concentration of less than about 250 milligrams of chloride per liter of culture medium.

14. A microfloral biomass comprising *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, wherein said *Thraustochytrium*, *Schizochytrium*, and mixtures thereof have a cell aggregate size less than about 150 microns.

15. The microfloral biomass of Claim 14, wherein said *Thraustochytrium*, *Schizochytrium*, and mixtures thereof have been grown in a culture medium containing sodium sulfate.

16. The microfloral biomass of Claim 14, wherein said *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, have all of the identifying characteristics of an organism selected from the group consisting of ATCC Nos. 20888 and 20889, and mutants thereof, wherein said mutants have an omega-3 HUFA content of at least about 0.5% dwt.

17. The microfloral biomass of Claim 14, wherein of said *Thraustochytrium*, *Schizochytrium*, and mixtures thereof have a sterol content of at least about 0.1% afdw.

18. The microfloral biomass of Claim 17, wherein *Thraustochytrium* and *Schizochytrium* have a cholesterol content of at least about 15% of the total sterol content.

19. The microfloral biomass of Claim 14, wherein said *Thraustochytrium*, *Schizochytrium*, and mixtures thereof have a cell aggregate size less than about 100 microns.

20. The microfloral biomass of Claim 14, wherein said *Thraustochytrium*, *Schizochytrium*, and mixtures thereof have a cell aggregate size less than about 50 microns.

21. A method to produce shrimp, comprising feeding microflora selected from the group consisting of *Thraustochytrium*, *Schizochytrium*, and mixtures thereof to larval shrimp, said microflora having a cell aggregate size less than about 150 microns.

22. The method of Claim 21, wherein said *Thraustochytrium*, *Schizochytrium*, and mixtures thereof have been grown in a culture medium containing sodium sulfate.

23. The method of Claim 21, wherein said *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, have all of the identifying characteristics of an organism selected from the group consisting of ATCC Nos. 20888 and 20889, and mutants thereof, wherein said mutants have an omega-3 HUFA content of at least about 0.5% dry weight.

24. The method of Claim 21, wherein said *Thraustochytrium* and *Schizochytrium* have a sterol content of at least about 0.1% afdw.

25. The method of Claim 24, wherein said *Thraustochytrium* and *Schizochytrium* have a cholesterol content of at least about 15% of the total sterol content.

26. The method of Claim 21, wherein said *Thraustochytrium*, *Schizochytrium*, and mixtures thereof have a cell aggregate size less than about 100 microns.

27. The method of Claim 21, wherein said *Thraustochytrium*, *Schizochytrium*, and mixtures thereof have a cell aggregate size less than about 50 microns.

28. A food product comprising:

a) microflora selected from the group consisting of *Thraustochytrium*, *Schizochytrium*, and mixtures thereof; and

b) a material selected from the group consisting of flaxseed, rapeseed, soybean, avocado meal, and mixtures thereof.

29. The food product of Claim 28, wherein said composition comprises between about 5% and about 95% by weight of said microflora.

30. The food product of Claim 28, wherein said composition comprises between about 5% and about 95% by weight of flaxseed.

31. The food product of Claim 28, wherein said composition comprises between about 5% and about 95% by weight of rapeseed.

32. The food product of Claim 28, wherein said composition comprises between about 5% and about 95% by weight of soybean.

33. The food product of Claim 28, wherein said composition comprises between about 5% and about 95% by weight of avocado meal.

34. The food product of Claim 28, wherein said food product is an extruded product.

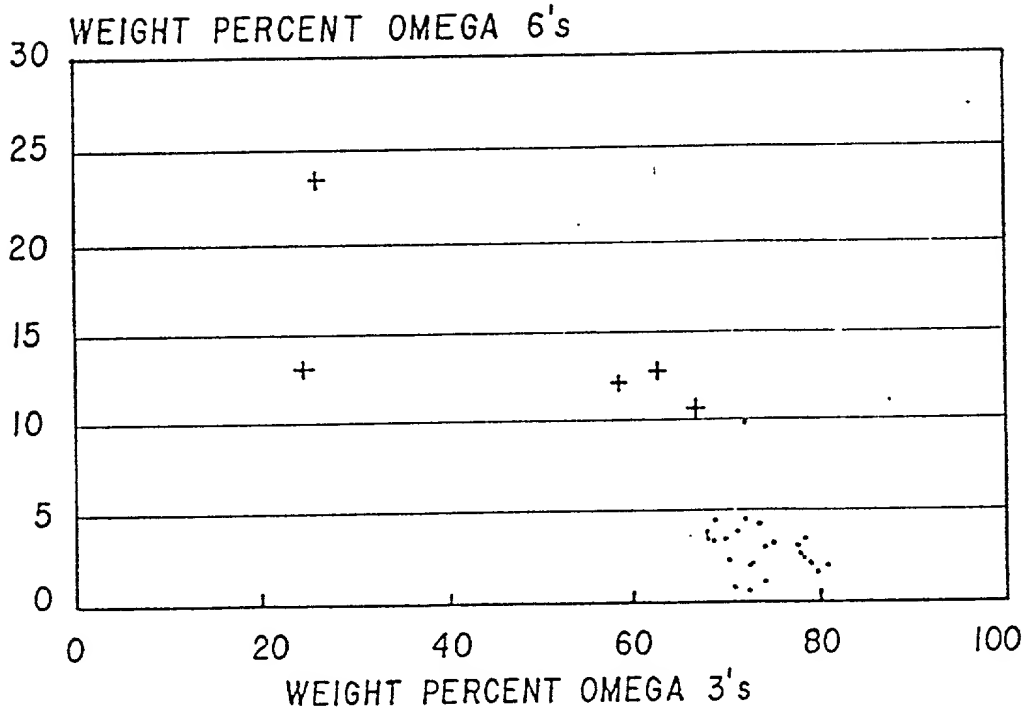
35. A method of aquaculture, comprising feeding microflora selected from the group consisting of *Thraustochytrium, Schizochytrium,* and mixtures thereof to organisms selected from the group consisting of larval shrimp, brine shrimp, rotifers and mollusks, said microflora having a cell aggregate size less than about 150 microns.

36. The method of Claim 35, wherein said *Thraustochytrium, Schizochytrium,* and mixtures thereof have been grown in a culture medium containing sodium sulfate.

37. The method of Claim 35, wherein said *Thraustochytrium, Schizochytrium,* and mixtures thereof, have all of the identifying characteristics of an organism selected from the group consisting of ATCC Nos. 20888 and 20889, and mutants thereof, wherein said mutants have an omega-3 HUFA content of at least about 0.5% dry weight.

ABSTRACT

Disclosed is a process for growing the microflora *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, which includes the growing of the microflora in fermentation medium containing non-chloride containing sodium salts, in particular sodium sulfate. In a preferred embodiment of the present invention, the process produces microflora having a cell aggregate size useful for the production of food products for use in aquaculture. Further disclosed is a food product which includes *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, and a component selected from flaxseed, rapeseed, soybean and avocado meal. Such a food product includes a balance of long chain and short chain omega-3 highly unsaturated fatty acids.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30
25
20
15
10
5
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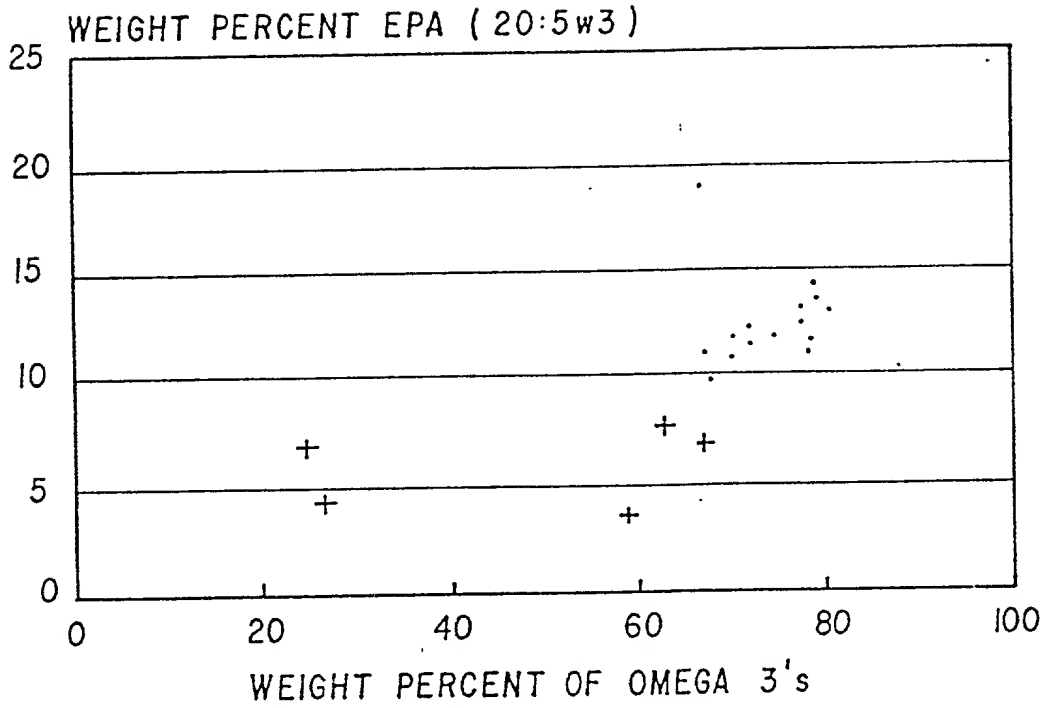
40
60
80
100
WEIGHT PERCENT OMEGA 3's
· NEWLY ISOLATED
+ PREVIOUSLY KNOWN

FIG. 1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20
15
10
5
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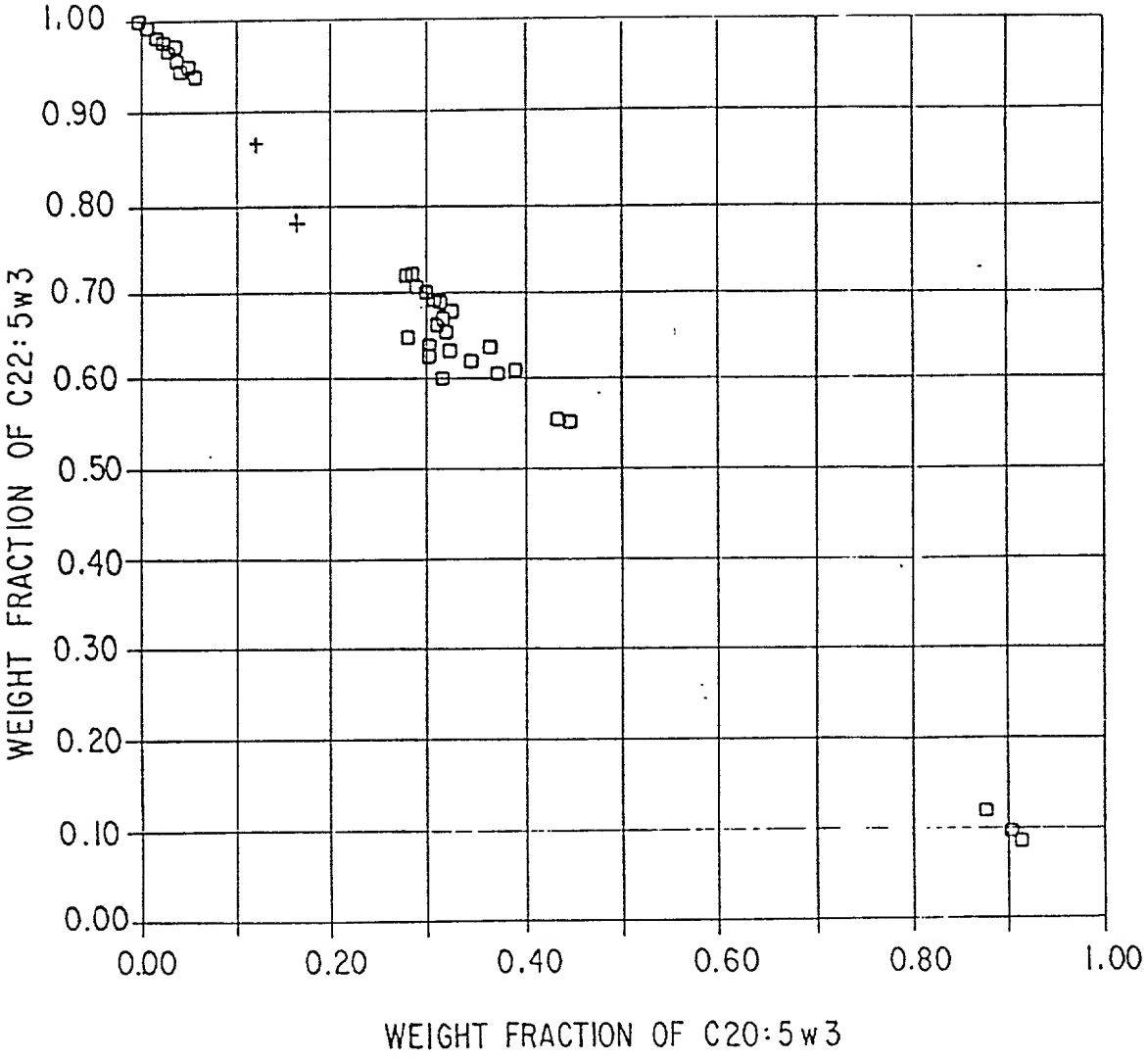
80
100
WEIGHT PERCENT OF OMEGA 3's
• NEWLY ISOLATED
+ PREVIOUSLY KNOWN

FIG. 2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WEIGHT FRACTION OF C22:5w3
0.00
0.20
0.40
0.60
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1.00
WEIGHT FRACTION OF C20:5w3
□ NEWLY ISOLATED
+ PREVIOUSLY KNOWN

FIG. 3

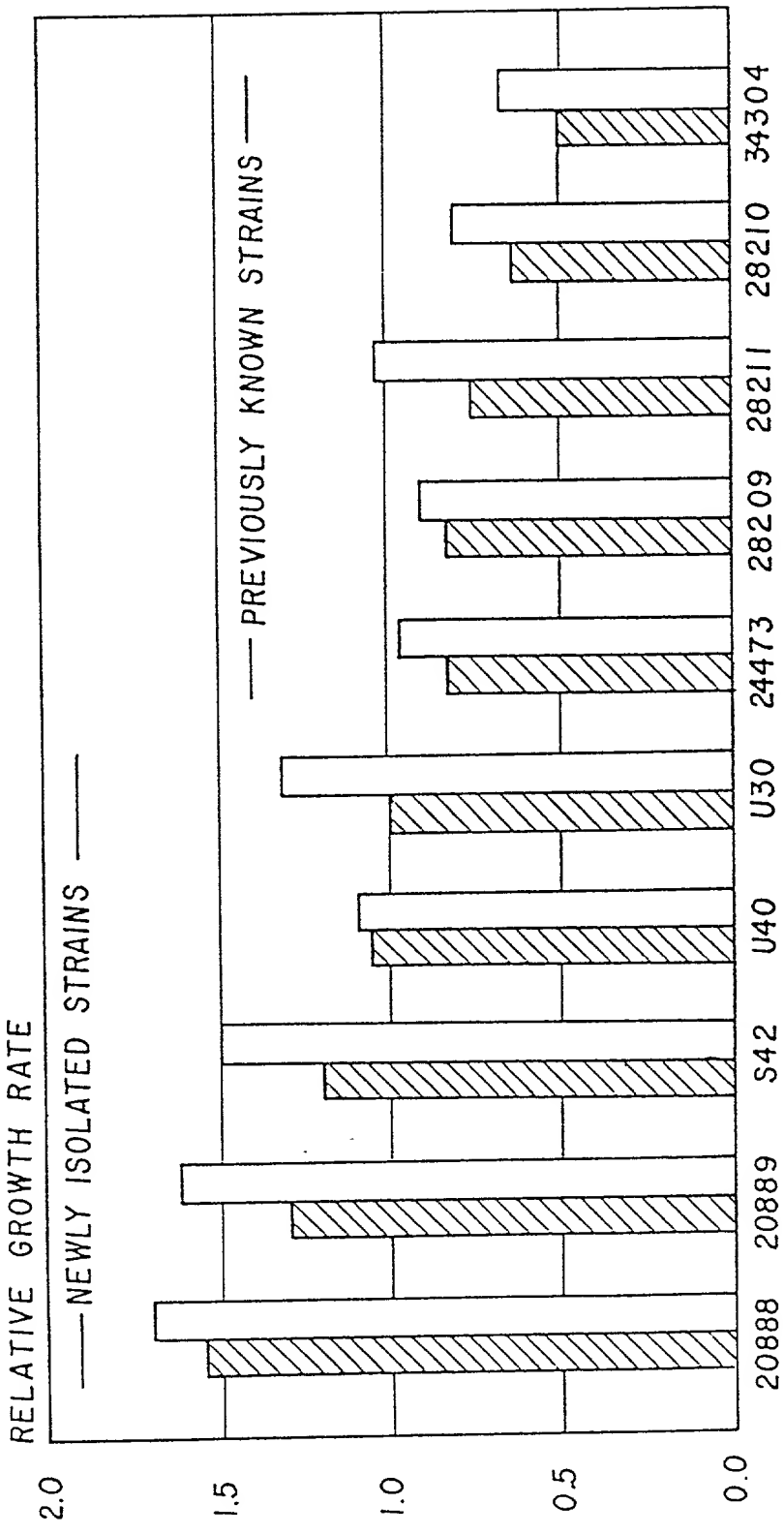
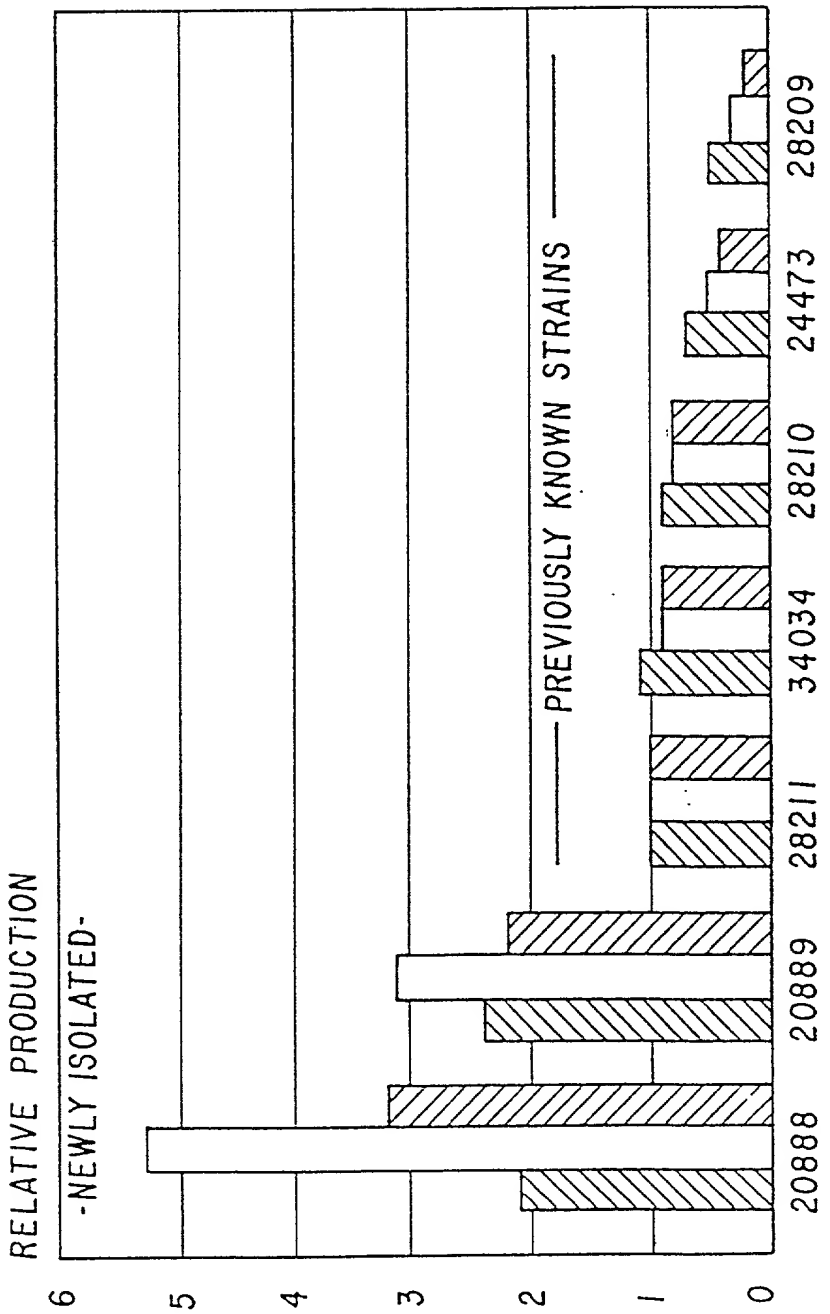

RELATIVE GROWTH RATE
2.0
1.5
1.0
0.5
0.0
NEWLY ISOLATED STRAINS
PREVIOUSLY KNOWN STRAINS
20888
20889
S42
U40
U30
24473
28209
28211
28210
34304
25 DEGREES C
30 DEGREES C

FIG. 4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-NEWLY ISOLATED-
PREVIOUSLY KNOWN STRAINS
6
5
4
3
2
1
0
20888
20889
28211
34034
28210
24473
28209
ASH FREE DRY WEIGHT
TOTAL FATTY ACIDS
OMEGA 3 FATTY ACID

FIG. 5

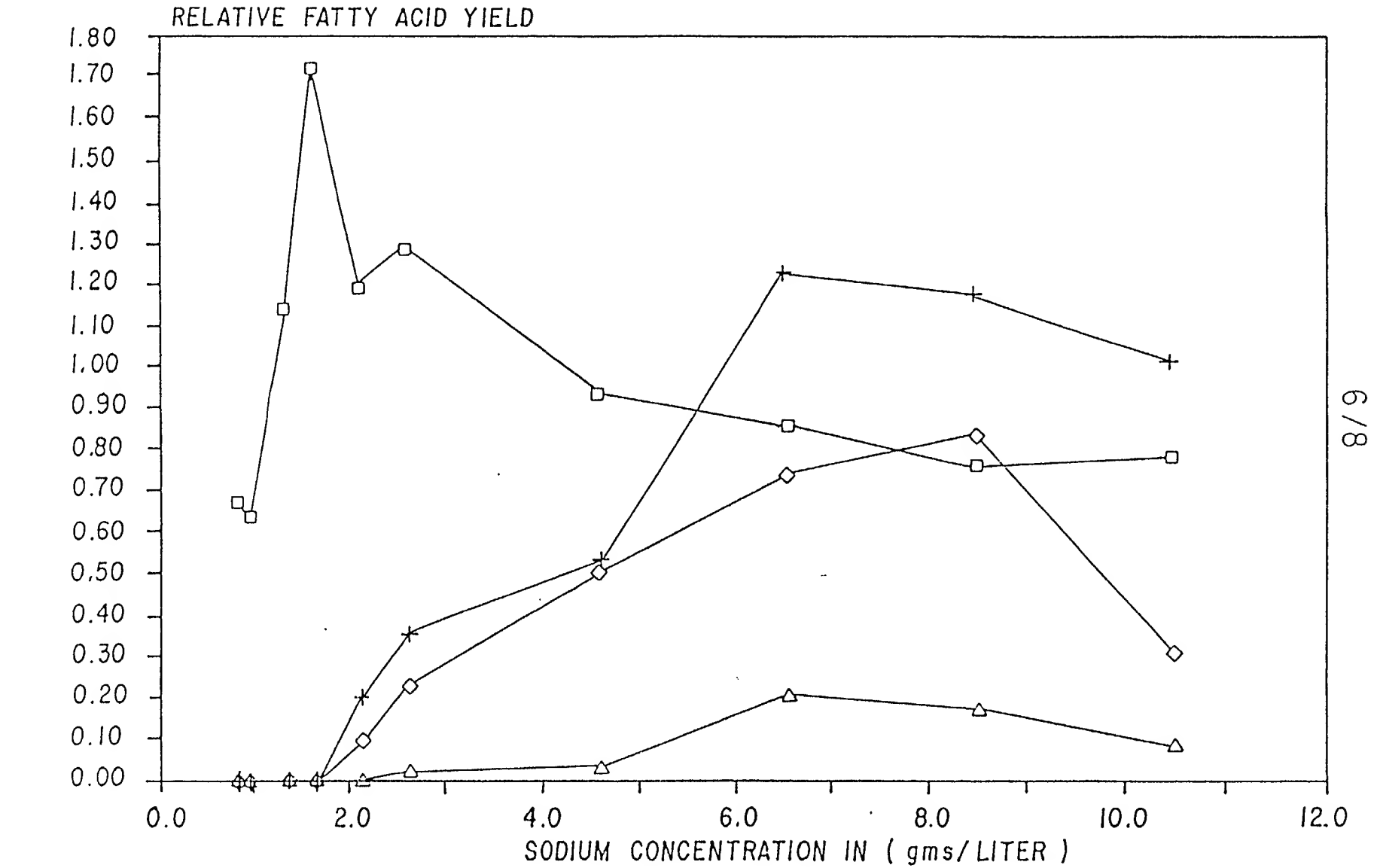
RELATIVE FATTY ACID YIELD
1.80
1.70
1.60
1.50
1.40
1.30
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.0
2.0
4.0
6.0
8.0
10.0
12.0
SODIUM CONCENTRATION IN (gms/LITER)
□ ATCC20888
◇ ATCC28211
+ ATCC20891
△ ATCC28209

FIG. 6

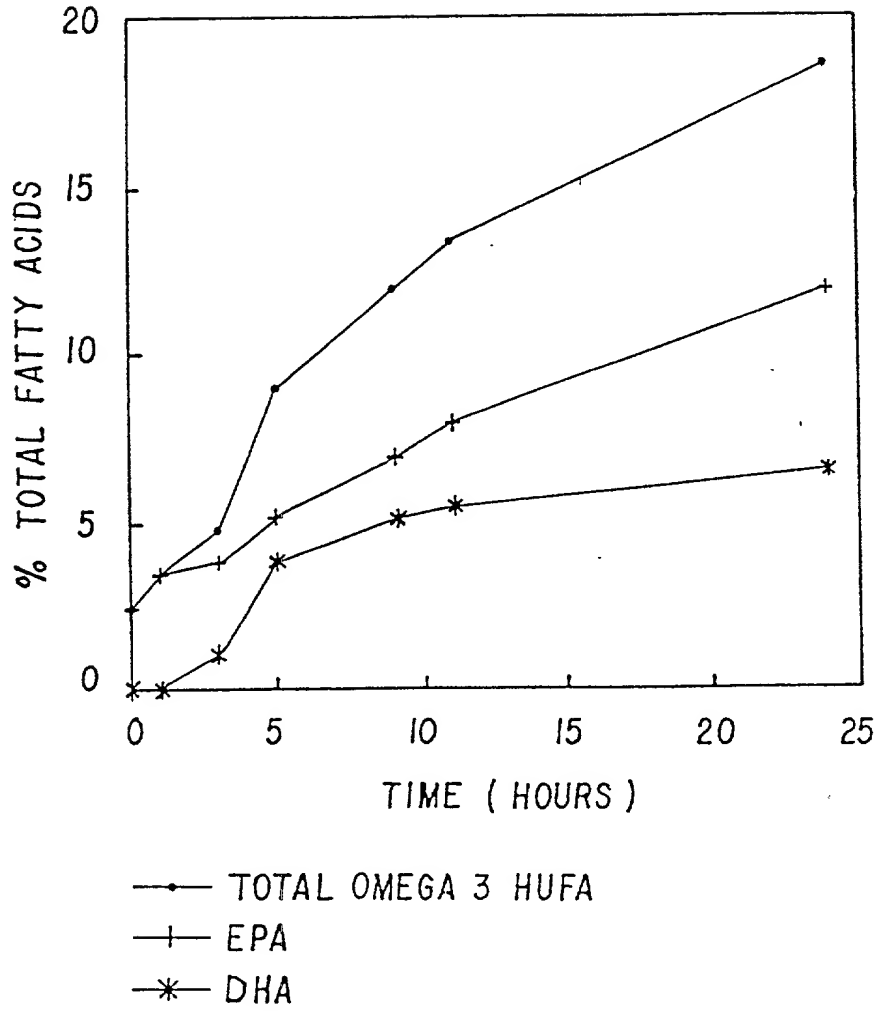
20
15
10
5
0
% TOTAL FATTY ACIDS
0
5
10
15
20
25
TIME (HOURS)
TOTAL OMEGA 3 HUFA
EPA
DHA

FIG. 7

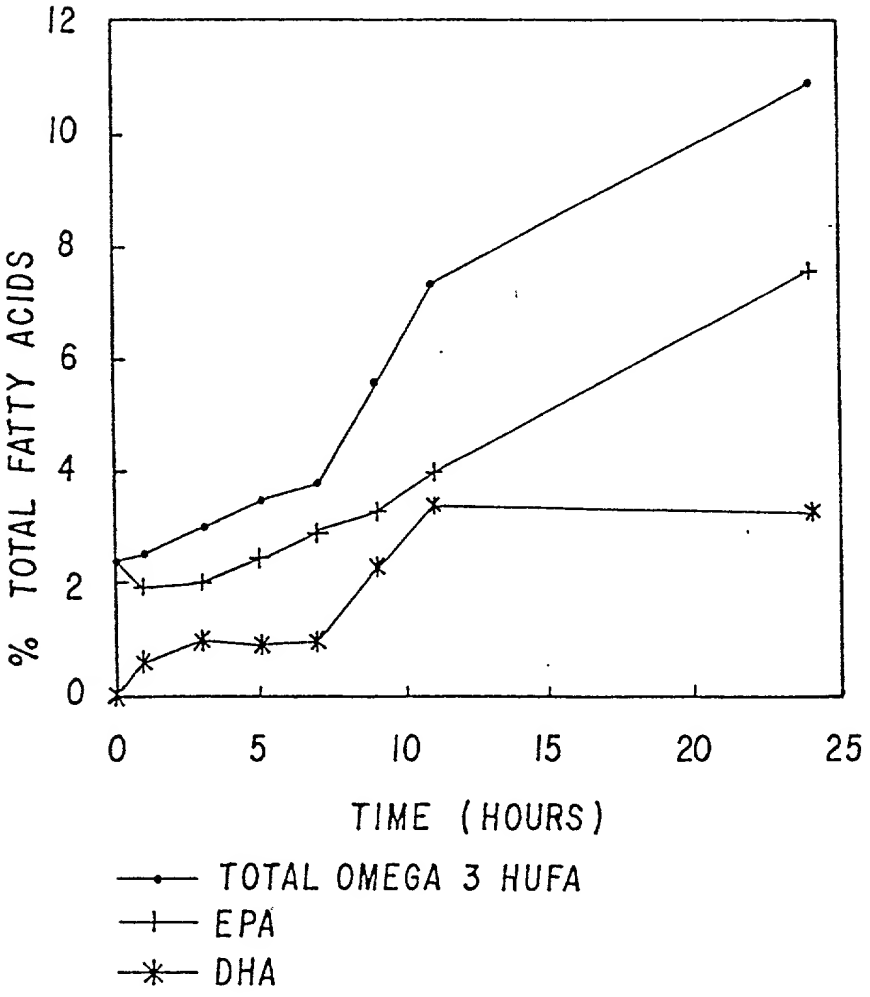
12
10
8
6
4
2
0
% TOTAL FATTY ACIDS
0
5
10
15
20
25
TIME (HOURS)
TOTAL OMEGA 3 HUFA
EPA
DHA

FIG. 8

RULE 63 (37 C.F.R. 1.63)
DECLARATION
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled "PROCESS FOR THE HETEROTROPHIC PRODUCTION OF MICROBIAL PRODUCTS WITH HIGH CONCENTRATIONS OF OMEGA-3 HIGHLY UNSATURATED FATTY ACIDS", the specification of which is attached hereto.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above, to the best of my ability. I acknowledge the duty to disclose information which is material to patentability in accordance with 37 C.F.R. 1.56(a) and (b) as set forth on the attached sheet indicated Page 3 hereof and which I have read.

I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
Number	Country	Day/Month/Year Filed	Yes	No

I hereby claim the benefit under 35 U.S.C. 120/365 of all United States and PCT international applications listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information material to patentability in accordance with 37 C.F.R. 1.56(a) and (b) which occurred between the filing date(s) of the prior application(s) and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status: patented, pending, abandoned
07/241,410	9/7/88	Abandoned
07/439,093	11/17/89	Abandoned
07/580,778	9/11/90	Issued as U.S. Patent No. 5,130,242
07/911,760	7/10/92	Pending

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1) Inventor's Signature William R Barclay Date Oct 16, 1992

Inventor's Name (typed): William R. Barclay

Citizenship: U.S.

Residence: 6395 Bruntwood Court
Boulder, Colorado 80303

Post Office Address*: Same as residence

*Complete Post Office Address in full if different from Residence, otherwise indicate that the Post Office Address is "SAME AS RESIDENCE."

37 C.F.R. §1.56(a) and (b)
DUTY TO DISCLOSE INFORMATION MATERIAL
TO PATENTABILITY

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is cancelled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

(1) prior art cited in search reports of a foreign patent office in a counterpart application, and

(2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

(1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or

(2) It refutes, or is inconsistent with, a position the applicant takes in:

(i) Opposing an argument of unpatentability relied on by the Office, or

(ii) Asserting an argument of a patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.*

*Note, 37 C.F.R. §1.97(h) states: "The filing of an information disclosure statement shall not be construed to be an admission that the information cited in the statement is, or is considered to be, material to patentability as defined in §1.56(b)."

RULE 63 (37 CFR 1.63)
DECLARATION
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe that, I am an original, first and sole inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled "A METHOD FOR REDUCING CORROSION IN A FERMENTOR", the specification of which is identified as Attorney File No. 2997-1-3-1-4 and attached hereto.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability in accordance with 37 CFR 1.56(a) and (b) as set forth on the attached sheet indicated Page 3 hereof and which I have read.

I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
Number	Country	Day/Month/Year Filed	Yes	No

I hereby claim the benefit under 35 U.S.C. 120/365 of all United States and PCT international applications listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information material to patentability in accordance with 37 CFR 1.56(a) and (b) which occurred between the filing date(s) of the prior application(s) and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status: patented, pending, abandoned
08/968,628	11/12/97	PENDING
08/461,137	6/5/95	PATENTED
08/292,490	8/18/94	PATENTED
07/962,522	10/16/92	PATENTED

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1) Inventor's Signature William R Barclay Date 10 DEC 99

Inventor's Name (typed): William R. Barclay

Citizenship: USA

Residence: 7356 Panorama Drive
Boulder, Colorado 80303

Post Office Address*: Same as Residence

*Complete Post Office Address in full if different from Residence, otherwise indicate that the Post Office Address is "Same as Residence."

37 CFR §1.56(a) and (b)
DUTY TO DISCLOSE INFORMATION MATERIAL
TO PATENTABILITY

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is cancelled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

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(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

(1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or

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(i) Opposing an argument of unpatentability relied on by the Office, or

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*Note, 37 CFR §1.97(h) states: "The filing of an information disclosure statement shall not be construed to be an admission that the information cited in the statement is, or is considered to be, material to patentability as defined in §1.56(b)."

POWER OF ATTORNEY

On behalf of OmegaTech Inc., a Colorado corporation having a principal place of business at 4909 Nautilus Court N., Suite 208, Boulder, Colorado 80301 being the assignee of and owning all right, title and interest in the invention entitled "A METHOD FOR REDUCING CORROSION IN A FERMENTOR", for which application for Letters Patent of the United States has been made by William R. Barclay, , said application being identified as Attorney File No. 2997-1-3-1-4 and executed on even date herewith, I, Mark A. Braman, President and CEO of OmegaTech Inc., hereby appoint David F. Zinger, Registration No. 29,127; Craig C. Groseth, Registration No. 31,713; Michael L. Tompkins, Registration No. 30,980; Sabrina C. Stavish, Registration No. 33,374; Todd P. Blakely, Registration No. 31,328; Lewis D. Hansen, Registration No. 35,536; Joseph E. Kovarik, Registration No. 33,005; Gary J. Connell, Registration No. 32,020; Wannell M. Crook, Registration No. 31,071; Douglas W. Swartz, Registration No. 37,739; Bruce A. Kugler, Registration No. 38,942; Robert R. Brunelli, Registration No. 39,617; Richard L. Hughes, Registration No. 31,264; Tejpal S. Hansra, Registration No. 38,172; Dana L. Hartje, Registration No. 40,638; Don D. Cha, Registration No. 40,945; Angela Dallas-Pedretti, Registration No. 42,460; Benjamin B. Lieb, Registration No. 42,801; Bradley M. Knepper, Registration No. 44,189; Theresa A. Brown, Registration No. 32,547; John C. Scott, Registration No. 38,613 and Miriam R. Drickman, Registration No. 42,499 of SHERIDAN ROSS P.C., 1560 Broadway, Suite 1200, Denver, Colorado 80202-5141, telephone number (303) 863-9700, as attorneys and agents for OmegaTech Inc. with full powers of substitution, association and revocation to prosecute the application and related U.S. and foreign applications and to transact all business in the United States Patent and Trademark Office and all foreign and international patent offices connected therewith.

By: Mark A. Braman

Mark A. Braman, President and CEO

Dated: December 10, 1999